# “Effects of biostimulators on growth and physiological reactions of vegetables - tested on cucumber (*Cucumis sativus L.*) “

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## List of acronyms

AAS –atomic absorption spectrometry

AC – air capacity (%)

BER - blossom end rot

BS – *Bacillus subtilis*

CEC – cation-exchange capacity;

cfu – colony forming unit;

CV - cylinder volume ($cm^3$)

D – density of the substrate

EC – electric conductivity ($mS*cm^{-1}$);

FB – portion of hard particles in the substrate sample

Fv/Fm – variable fluorescence/maximum fluorescence;

g – gram

GPB - growth–promoting bacteria;

HA - humic acid;

HM – heavy metals;

l - liter

LG - air content ($cm^3$);

MOMP - major outer membrane protein;

PAR – photosynthetic-active radiation;

PGPB – plant growth promoting bacteria;

ppm – Parts per million (concentration);

PSII – Photosystem II;

PV - pore volume (%);

PW – value shown by air-pycnometer;

SIR - substrate-induced respiration;

$W_1$, $W_2$, $W_3$ - cylinder masses after different manipulations (g);

WC- water holding capacity (%);

WG - water content in the substrate ($cm^{-3}$);

µm – micrometer;

**Contents**

# 1 Introduction

Plant production in hydroponical systems is under scientific investigation since early sixties of the last century (BENOIT and CEUSTERMANS 1994). Gained systematic knowledge about benefits and shortcomings of soilless culture allowed its use in commercial vegetable growing (LEEMAN *et al.,* 1995). From the three cultivation systems (substrate culture, water culture, aeroponics) for plant production, the substrate culture is mostly used because of its capability to provide sustainable production of fruits and vegetables around the year including the regions with limited water availability, that renders the horticultural production impractical or not possible at all.

Soilless culture or hydroponics is in position to contribute to sustainable production of vegetables through adoption of most sufficient growing conditions with regard to plant's requirements in nutrient elements, water supply, climatic conditions as well as modern managerial practices (LEEMAN *et al.,* 1995). In soilless culture and hydroponics, an optimization of growing conditions can be achieved through utilization of appropriate growing substrates, nutrient solution and optimal growing conditions. The role of suboptimal growing factors – optionally referred to as "stress factors", contribute to reduction of horticultural produce (OLYMPIOS, 1992). Changes in the horticultural output in both quality and quantity of the horticultural products can occur as a result to the changes in the buffering capacity of the horticultural substrates, pH and EC change in the rhizosphere of the horticultural crops (VERDONCK and GABRIELS, 1988). The factor that influences sustainability of the horticultural production is a creation of the optimal growing conditions during the whole vegetation period. The term "growing conditions" generalizes description of biotic and abiotic factors that exercise their influence on cultivar's growth and development. The growing conditions that are deviating from optimal intensity or quantity for the plant are called stress factors (SCHULZE *et al.,* 2002). Plants under stress conditions adjust their physiology as a response function to the suboptimal factors. This adaptation of plant's physiology to the specific suboptimal growing condition effects the yield formation of the horticultural crops. Hence, the optimization in the horticultural practices is achieved by creation of the physiologically optimal growing conditions. The optimization of the growing conditions is achieved by adaptation of the appropriate plant management techniques. Utilization of different naturally-occurring bioactive substances such as humates, microorganisms leads to sustainable growth of the horticultural crops (De KREIJ and HOEVEN, 1997). A range of naturally-occurring and artificially-derived

compounds with a range of biostimulating properties is used for the optimization of the plant growth (SAPUNDJIEVA *et al.,* 1997).

Humic acids and humic substances as a whole are biological polymers, products of biodegradation of organic material. Humic substances enhance plant growth both directly and indirectly. Physically, they promote good structure of some organic substrates and increase the water holding capacity of almost all horticultural substrates (FONTENO *et al.,* 1981). Biologically, they influence the activities of the substrate microorganisms. Chemically, they serve as an adsorption and retention complex for inorganic plant nutrients. Nutritionally, they are sources of nitrogen, phosphorus, and sulphur for plants and microorganisms. These effects can increase the productivity of horticultural substrates used in the greenhouses. Commercially-available humic compounds applied to the horticultural substrates do not contribute significant quantities of nutrients for plants. The indirect effects of these materials on substrate fertility and its general condition can be significant. Micronutrients of the substrates are more available to plants in the presence of humates (FONTENO *et al.,* 1981). Inorganic iron compounds, for instance, are very unstable in substrate and tend to become insoluble and unavailable for plant uptake. Humate can incorporate iron into chelated complexes, maintaining its availability to plants, although still in insoluble form. Availability of the phosphor for the plant root system can be improved through immobilization during the reactions with iron and aluminum. This leads to complex creation between phosphorous and organic matter (Mc CARTHY *et al.,* 1990). Chelating agents can break the iron or aluminum bonds between the phosphate and organic matter, releasing phosphate ions into solution (HAJRA and DEBNATH, 1985). The humates can be applied in the soil culture for the soil amendment and therefore can play very important ecological role (YONEBAYASHI *et al.,* 1988). The role of the humates in the soilless culture is limited to the experimental level (HOANG, 2003).

Application of lactates in agriculture remained confined to the country of their invention - Bulgaria. The lactates in the horticultural system in the form of the foliar fertilizer were used on the experimental level in Germany (BOEHME, 1999). The lactates can be used for creation of optimal growth conditions in the root area of horticultural crops. This approach for creation of the sustainable growing conditions is based on the lactates' ability to form chelate complexes (CHEN *et al.,* 1998). This results in increased productivity of the horticultural plants (CLAPP *et al.,* 2001). The use of the lactates in the root area of the plants, improves the supply of micronutrients. This fact facilitated creation of the test program – part of this scientific

undertaking. Application of the lactates in different combinations with humates and *B.subtilis* – ubiquitous microorganism is the subject of the current scientific piece.

Antagonism between different microorganisms is used for the purpose of the pest and disease control. It is a cornerstone element of the biological production of horticultural crops. The selection process of the particular microorganism is based on identification of the naturally occurring antagonists (GROSCH *et al.,* 1999). Such an antagonist, who is both well researched as well as proved efficient in the practical horticulture is *Bacillus subtilis* strain (GROSCH *et al.,* 1999). Application of rhizobacteria in hydroponics with their antagonism against malicious microorganisms has different beneficial effects in adoption of the optimal growing conditions for the horticultural plants (BROADBENT *et al.,* 1977). Different studies on utilization of *Bacillus subtilis* and its different strains showed that its interaction in the growing systems is beneficial for creation of the optimal cultivation conditions (BOCHOW *et al*., 2001; BAIS *et al.,* 2004; BÖHME *et al.,* 2005).

*Bacillus subtilis* accommodates itself on the root system of the host plants and the area around the plant's root (BOCHOW, 1989). After application of *Bacillus subtilis*, it resides in the substrate for many years and do not lose its capacity of natural antagonist but at the same time it is effective only when it is in active form. To sustain its active statues of the microorganism, different conditions are to be observed. Different factors influence development of the antagonistic potential. The water availability, substrate temperature and availability of nutrient elements in different forms are the factors that can sustain optimal substrate conditions.

Humates, lactates and *B.subtilis,* because of their different nature and hence different chemical, physical and physiological characteristics they have specific action spectrum that limits their overall positive effect on plants. Combination of these all three biostimulating substances contributes to increased efficiency of all three components and improves productivity of the horticultural crops. This is a solution against recurring suboptimal abiotic factors. This research focuses on application of biologically active substances with the purpose of creating a biostimulating mixture of humates, lactates and *B.subtilis* with wide activity spectrum that can sustain development of horticultural crops over vegetation period and insure formation of high quality yield. The research program presented in this script was imbedded in the scientific activity of the department for horticulture.

# 2 Review of literature

## 2.1 Protected plant cultivation

### 2.1.1 Hydroponical systems – their advantages and disadvantages

Optimization of hydroponical technique forestalls disadvantages which restrict the further expansion of hydroponical cultivation methods. Targeted optimization of nutrient supply in commercial hydroponics is a primary objective of the research work related to soilless culture (De KREIJ and HOEVEN, 1997). In practical horticulture one can observe constant diversion from optimal growing condition for a particular crop caused by variability of growing parameters and their clustered influence on plant's development. Hydroponical technology can be characterized through specific requirements it imposes on the cultivation of horticultural plants. Hydroponics gives an advantage of full control over the most growing factors needed for plan's development. These growing factors interact between each other and the plant. The level and intensity of these interactions influences the formation of specific climatic and microclimatic conditions within a greenhouse. The range of the microclimatic conditions created by the plants in their development influences the immediate cultivation environment in the greenhouse. Diseases, pests, availability of nutrient elements, activity of microbial community in the substrate, suboptimal growing conditions – any of these factors can influence plants growth and productivity. Considering horticultural plants as an entity – intertwined and interconnected group of biological mechanisms that are functioning in many aspects far beyond our comprehension, we can assume that a status of one plant effects the others within given ecosystem. The pros and cons of the hydroponic culture in comparison with the soil culture can be summarized as follows:

1. **Balanced nutrient solution supply**. Diligent control of all components of the nutrient solution and its supply to the plants creates optimal conditions for controlling the development of the plants in the course of the whole vegetation process. Sampling of the nutrient solution can be done and based on its results, one can replenish those nutrient elements that were leached or consumed by the plants. Optimization of the nutrient supply is achieved by adopting an optimal combination of the nutrient elements and their concentration in the nutrient solution.
2. **Natural conditions and demand for regional product.** Crops can be cultivated in places and regions where natural conditions render production of crops impossible. For instance, seasonal fluctuations of temperature and photosynthetic-active radiation make around-the-year production of most horticultural crops impossible. Another factor, the difference in soil fertility and the level of technology for sustainable agricultural production. One of the most important factors that contributes to preservation and development of horticultural production despite relatively high costs is the local

demand for regional products. Consumers are willing to pay for products from their region despite the higher price of the produce.

3. **Aeration.** Hydroponical production makes it possible to create optimal aeration of the root system of the plants. The root system of the plant needs oxygen for respiration, which influences nutrient absorption (ADANI *et al.,* 1998). Utilization of different horticultural substrates provides an opportunity to adopt the optimal conditions for gas exchange in the root area of the plants. In the case of liquid culture, the aeration can be achieved in several ways: continuous aeration of the nutrient solution, continuous flowing of the nutrient solution (BÖHME *et al.,* 1993). Under practical conditions this parameter is often a subject of drastic fluctuations (KREBS *et al.,* 1998).
4. **Water supply.** In many regions, availability of water is a limiting factor of agricultural production, which makes horticultural production a choice that paves the way to more rational water distribution. The quantity of water used for horticultural produce is lower then that for traditional soil cultivation (OLYMPIOS *et al.,* 1994).
5. **Disease control.** Most horticultural substrates nowadays come in practical use after sterilization. The sterilized substrates decrease possibility of root disease recurrence (GULER *et al.,* 1995). Additional sterilization is performed between different fruit rotations. Utilization of the new cultivars provides another possibility to decrease occurrence of the diseases. Modern horticultural practice includes utilization of biological agents for control of pests and diseases (GULER *et al.,* 1995).
6. **Plant productivity.** Hydroponic production often brings higher results in terms of plant's productivity. Major growth factors are under control and can be managed according to plant's stage of development or individual biological requirements.

Beneficial effects of the soilless culture listed above contribute to sustainable production of horticultural produce. The variety of suboptimal growing conditions may arise as a result of multipronged changes in substrates, plants and nutrient solution. Every parameter has its own dynamic and interconnectivity with other growing parameters. Physical properties of the horticultural substrates change with time as a result of their interaction with plants, nutrient solutions, microorganisms and these changes are not reversible (CARLILE *et al.,* 1984). EC and pH values are exposed to even more drastic fluctuations and can represent a source of stress in their extreme values.

### 2.1.2 Horticultural substrates and their special effects

Horticultural substrates are different by nature and can be classified as those of organic and inorganic origin. The substrates can be otherwise referred to as root media, soils, and growing media. There several different functions that can be fulfilled by the substrates:

1. Physical support for plants;
2. Water retention in a form accessible to plants;
3. Gas exchange between the root, atmosphere and microbial community;
4. Exchangeable accumulation of plant nutrients;
5. Disease suppression and support of microbial communities important for the plants.

Depending on the substrate type and growing system these functions are not necessarily implemented at any given time. Most used inorganic and organic substrates are listed in the table 2.1. Important parameters of substrate for plant growing are pore volume, air and water capacity, cation exchange capacity.

**Table 2.1** Soilless cultivation systems in hydroponics (SCHWARZ, 1995)

| **Solution culture** | **Aggregate systems** | | |
|---|---|---|---|
| | **Inorganic media** | | **Organic media** |
| | **Natural media** | **Synthetic media** | |
| Static solution* | Sand | Foam mats | Sawdust |
| Circulating solution* | Gravel | Plastic Foam | Bark |
| Aeroponics | Rockwool* | Hydrogel | Wood chips |
| | Glasswool | | Peat* |
| | Perlite* | | Sheep wool* |
| | Vermiculite | | Coir* |
| | Pumice | | |
| | Expended clay | | |
| | Zeolite | | |
| | Volcanic Tuff | | |
| | Sepiolite | | |

*Substrates used in current study

**Perlite** has very good physical characteristics, and high potential to be used as a closed water efficient system in areas with good quality water or as an open system where poorer quality water dictates this. Several systems have been developed which use perlite as a substrate. These are described by (WILSON, 1980; OLYMPIOS, 1992). In the literature it is shown that perlite is superior to other substrates for crop production. The comparison of perlite, rockwool and sand in open systems and their influence on the yield and quality of sweet melon was evaluated. The results show that perlite gives similar values as rockwool and has the great advantage of the much lower cost (GULER *et al.,* 1995). The another experiment with the natural pumiceous

perlite and row perlite produced similar results as horticultural perlite in both growth and production, when tomatoes were grown in open systems on these substrates (OLYMPIOS *et al.,* 1994).

**Rockwool.** Good results have been obtained with rockwool in many countries and examples of using this material in commercial greenhouses are well known (Holland, France, U.K., Denmark, *etc*.), all having good control on the environmental growing factors and the application of nutrient solution (OLYMPIOS *et al.,* 1994 and GULER *et al.,* 1995). At the same time application of rockwool in horticulture made it possible to broaden application of hydroponics throughout the Europe. Rock wool is produced by burning a mixture of coke, basalt and limestone at a temperature of 1600°C. The mixture liquefies and the liquid is spun to form fibers. Rockwool has a negligible cation-exchange capacity. Rockwool cubes are usually used for propagation purposes in hydroponic culture.

**Peat.** Peat is a very common substrate for modern horticulture. In total, approximately 20 million $m^3$ of horticulture peat are processed and traded in Europe (SCHMILEWSKI, 1997). For more than 30 years organic substrates (peat, moss, etc.), have been the dominating bulk material in substrates for growing plants. In many countries that have horticultural industry, peat is a substrate that is used very broadly in the greenhouses. There several different types of peat that come from the different plant source and have different degree of decomposition.

1. Sphagnum peat moss is light to medium brown in color, is formed primarily from Sphagnum peat, and is the least decomposed of the general category of peat. It decomposes relatively slow so nitrogen tie-up does not occur. It has the heist water holding capacity of the all peats – 60% of its volume in water. Its pH of 3.0 to t.0 is the lowest of all the peats and its cation-exchange capacity of 90-140 meq $100g^{-1}$. This type of peat is the most common in horticulture.
2. Hypnum peat moss is darker in color than sphagnum peat, and it is composed primarily of hypnum moss. Its texture is finer that Sphagnum peat and it has pH of 5.0 to 5.5. Cation-exchange capacity of the Hypnum moss ranges from 100 to 200 meq $100g^{-1}$.
3. Reed-Sedge peat is brown to red in color and is formed from a variety of plant material (i.e. reeds, sedges, grasses and cattails). Although it can be obtained in different degrees of decomposition, it is usually more decomposed than sphagnum and hypnum peat. Its water holding capacity is lower than that of sphagnum and hypnum peats, and it has a pH that ranges from 4.0 to 7.5. Cation-exchange capacity of this peat type is usually between 80 to 100 meq $100g^{-1}$.
4. Peat humus is dark brown to black in color and is the most highly decomposed of all the peats. It is usually derived from hypnum or reed-sedge peat. The plant remains are well decomposed and cannot be distinguished. This type of peat can contain significant

amounts of mineral soil. pH values varies between 5.0 to 7.5. Cation-exchange capacity of the peat humus is 160 to 200 meq $100^{-1}$.

If the soilless system is closed, then more frequent chemical analysis of the solution is required (BENOIT and CEUSTERMANS, 1994). The favorable factors which ensure that peat continues to be the material of choice in both professional and hobby horticulture can be summarized as:

1. The stable cellular structure which ensures balanced air and water holding properties throughout the life cycle of the growing media.
2. Low bulk density ensuring ease of handling and transportation.
3. Low pH, which permits accurate liming to optimal pH ranges for all crop types.
4. Low nutrient content, ensuring no salinity problems and ease of adjustment of nutrient levels for all applications by either liquid feed of soluble fertilizer or controlled release fertilizers.
5. Free of pathogens, pests, seeds of other plants.
6. Ready availability, consistency of quality and competitive pricing.

**Coir.** Coconut coir is a waste product of coconut industry. This material is produced in Sri Lanka, the Philippines, Indonesia, Mexico and other parts of South America. Coir is obtained by grinding the coconut husk and screening the long and medium length fibers. Coir dust is a common substrate used in horticulture that has been proved to have air and ion exchange capacity. It can absorb ions such as $NH_4^{4+}$ and $N\text{-}NO_3^{3-}$ preventing their leaching into the environment. At the same time it is often recommended to increase concentration of nitrogen in the nutrient solutions in combination with coir dust substrate. The reason for this is its ability to retain nitrogen ions (ADANI *et al.,* 1998). The pH of coir can range from 5.6 to 6.9. The electrical conductivity of this material varies from 0.3 to 2.9 $mS*cm^{-1}$. With a cation-exchange capacity of 39 to 60 meq $100^{-1}$ coir provides for nutrient-holding capacity in the substrate. Coconut coir typically contains higher levels of mineral elements than sphagnum peat. Coir has a similar or slightly lower bulk density and air-filled pore space than most sphagnum peats.

**Sheep wool.** Sheep wool is a byproduct of sheep husbandry. This substrate is a perspective for the regions with sheep breeding farms. The substrate is not that common in the horticultural production as the other horticultural substrates. Application of this substrate in combination with other organic and inorganic substrates can improve their aggregate physical and comical properties. The substrate in the form of pellets can also be used for soil amendment purposes.

The horticultural production is largely dependent on different kinds of substrates (ADANI *et al.,* 1998). Physical properties of the substrates used in this study described in table 2.2.

**Table 2.2** Qualitative description of different horticultural substrates

| Substrate | Bulk density | Water holding capacity | Porosity | Stability of structure | pH | EC | Nutrients | Pathogens, Pests, weeds |
|---|---|---|---|---|---|---|---|---|
| **Perlite** | ++ | + | ++ | ++ | 0 | 0 | --- | ++ |
| **Rockwool** | + | + | 0 | ++ | 0 | 0 | --- | 0 |
| **Coir** | ++ | + | + | + | 0 | -- | 0 | 0 |
| **Peat** | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| **Sheep wool** | -- | --- | + | --- | ++ | ++ | ++ | --- |

++ very favorable; + favorable; 0 neutral; -- unfavorable; --- very unfavorable.

The materials mentioned above can be viewed as substrates or as components for substrate preparation. If a special set of physical properties is required, then these materials can be mixed together in different proportions. Components and ratios of the components can be adjusted to obtain the desired properties of the final substrate to meet the requirements of particular plant.

### 2.1.3 Plant stress and stress quantification in horticulture

#### 2.1.3.1 Influence of climate conditions

Horticultural production is a dynamic system and suboptimal growing conditions might occur during vegetation. A decisive factor here is the ability of the plants to adopt their physiological reactions to these stress factors. This stress resilience can be triggered either on the chromosome level or by application of certain substances of biostimulating nature. Addressing the problem of plants productivity in suboptimal growing conditions and evaluation of plant's physiological responses to application of humates, lactates and *B.subtilis* contributes to significance of this study.

In order to investigate effects of different combinations of such substances as humates, lactates and *B.subtilis* it is necessary to take into consideration several factors that are incident to horticultural production. In hydroponics, investigations about the effect of such mixed biostimulators are scares until now. At the same time application of biostimulating substances is often limited by biotic and abiotic stresses (KREBS *et al.,* 1998). Cultivation of horticultural plants under hydroponic culture can be challenged by suboptimal growth conditions during the vegetation period. In previous investigations we found beneficial effects in hydroponics of the gram-negative rhizobacteria *Bacillus subtilis* FZB 24® regarding the reduction of salt stress (BOEHME, 1999). To address this risk and improve sustainability of horticultural production a multipronged approach is needed.

### 2.1.3.2 Situation in the rhizosphere

Hydroponical production of vegetables is inevitably connected with particular substrate. For soilless culture, however, it is extremely critical to maintain stable values of pH, general availability of nutrient element in the substrate. Effects of *Bacillus subtilis* FZB 24® against fungal and bacterial diseases are also proved (LOEFFLER *et al.,* 1986; SCHMIEDEKNECHT *et al.,* 1998; GROSCH *et al.,* 1999). In previous researches it was proved that application of specific combinations of biostimulating agents is capable to see plants through critical periods of vegetations specifically during transplanting, flowers setting, fruits developments (BOEHME *et al.,* 2005). Beside microorganisms, also organic substances with different chemical composition can be used as biostimulators, e.g. humates and lactates. Also for these substances growth stimulating and stress-reducing effects could be shown in hydroponics (BOEHME, 1999; BOEHME *et al*., 2000; HOANG, 2003). Humates are known as main components of soil fertility. They have so far no importance in hydroponics. However, some very interesting effects of humates are described concerning their stimulating effect on nutrient uptake (FORTUN and LOPEZ, 1982; TATTINI *et al.,* 1989), counteracting salt and drought stress as well as temperature stress. The positive effect of humates on availability and uptake of nutrients like calcium, magnesium, and phosphorus due to chelating should be stressed. Chelating agents in form of humates and lactates may suppress the growth of plant pathogens by depriving iron and hence favorable plant growth. Identification and quantification of stress at an early stage could help to counteract it by changing growing conditions.

### 2.1.3.3 Microbial activity in hydroponics

Assessment of microbial activity in the substrates is an important characteristic for decision making about status of microbial community (CARLILE *et al.,* 1991). Microbiological activity can be interpreted as $CO_2$ efflux from substrates. Moreover, ANDERSON and DOMSCH (1978) described relationships between environmental conditions, such as pH, and the microbial biomass of forest soils. Others identified interdependencies between availability of organic substance in the soils and microbial activity, (GARCÍA, 2003). Horticultural practices usually operate with horticultural substrates and not soil; nevertheless the same methodology can be applied for analytical assessment of microbial activity.

Current methods of substrate evaluation that was used for the assessment of microbial activity hinges on the principle described by ANDERSON and DOMSCH (1978). The substrate induced respiration (SIR) is based on determination of the substrate's respiration after addition of

glucose. The method facilitates quantification of microbial activity and thus microbiological status in the substrates. The basal respiration represents $CO_2$ efflux from the microbial community of the substrate without addition of glucose. Total volume of $CO_2$ efflux within certain period of time (which is individual for every substrate) is interpreted as integral respiration.

Addition of glucose to the substrates triggered glucose-induced respiration. Glucose used as a preferable source of energy for microorganisms and creates conditions for respiration – sign of active metabolism of microorganisms. Addition of glucose also causes growth of microorganisms than finds its expression in incremental $CO_2$ dynamics.

The basal and glucose-induced respirations as function of microbial activity in the substrates inevitably interact with the root system of the plant. Depending on composition of microbial community as well as its metabolic activity influences the rhizosphere of test plants. Different basal and glucose-induced respirations are attributed to differences in substrate nature.

#### 2.1.3.4 hemical situation and changes in the substrate

Different physical properties of substrates lead either to leaching or accumulation of nutrient elements. Nitrogen is an element that is used in metabolic processes of both microorganisms and plants. This fact brings up an assumption that development and productivity of plants in horticultural production may depend on both nutrient availability and status of microbial community. In this research variants with peat and coir that gave maximum result in terms of productivity of cucumber plants have also accumulated highest concentration of $N\text{-}NO_3$. RUPPEL *et al.,* (2007) found that nitrogen availability decreases prokaryotic diversity in sandy soils what in turn can be translated onto substrates of inorganic nature such as perlite, rockwool. An introduction of biologically active components during the vegetation period of horticultural plants and especially at most critical development stages of the plants can change situation within the root area and in the substrate at large.

## 2.2 Use of biostimulators for improving the growing conditions

Humates positively influence root system growth and nutrient element uptake. Application of the humates can lead to accumulation of the nutrients in the root area of the plants and therefore can influence development of the rhizospheric microorganisms.

### 2.2.1 Effects of microorganisms as plant strengthener

Complex plant-microorganism interactions in the rhizosphere are responsible for a number of intrinsic processes such as carbon sequestration, ecosystem functioning, and nutrient cycling (SINGH *et al.,* 2004). Availability, composition and quantity of microorganisms in the soil influence the ability of a plant to obtain nitrogen and other nutrients. Different interactions between plants and substrates in complex with different abiotic and biotic factors results in a deposition of secondary metabolites into the rhizosphere that can promote or inhibit the growth of specific microorganisms (KEHLENBECK *et al.,* 1994). This rhizodeposition consists of small-molecular weight metabolites, amino acids, secreted enzymes, mucilage, can range from less than 10% of the net carbon assimilation by a plant to as much as 44% of a nutrient-stressed plant's total carbon (GRAYSTON *et al.,* 1998). Available microorganisms are in position to utilize this ample energy source during their lifecycle, thereby implying that selective secretion of specific compounds may encourage beneficial symbiotic and protective relationships, whereas secretion of other compounds inhibit pathogenic associations providing plants sufficient conditions for growth and development (HOFFLAND *et al.,* 1992). Plant-bacteria interactions can positively influence plant growth through different mechanisms, including fixation of atmospheric nitrogen by different classes of proteobacteria (MOULIN *et al.,* 2001), increased biotic and abiotic stress tolerance imparted by the presence of endophytic microbes (SCHARDL *et al.,* 2004), and direct and indirect advantages imparted by plant growth promoting rhizobacteria (GRAY and SMITH 2005). Bacteria can also positively interact with plants by producing protective biofilms or antibiotics operating as biocontrol against potential pathogens that contributes to formation of positive microbial community within the root area of the plant (BAIS *et al.,* 2004.) Soil bacteria are also taking part in degrading plant- and microbe-produced compounds in the soil that can be allelopathic or even toxic to next generations of microorganisms as well as higher plants (HOLDEN *et al.,* 1999).

However, rhizosphere bacteria can also have detrimental effects on plant health and survival through pathogen or parasite infection. Secreted chemical signals from both plants and microbes mediate these complex exchanges and determine whether an interaction will be malicious or benign. Root colonization is important as the first step in infection by soil-borne pathogens and beneficial associations with microorganisms (PATTERSON *et al.,* 2000).

The "rhizosphere effect," (HILTNER, 1904), assumes that many microorganisms are attracted to nutrients exuded by plant roots. Hiltner observed that the number and activity of microorganisms increased in the vicinity of plant roots. That increment in microbiological activity is attributed to

efflux of secondary metabolites by the root system of the plants. However, in addition to providing a carbon-rich environment, plant roots initiate cross talk with soil microbes by producing signals that are recognized by the microbes, which in turn produce signals that initiate colonization (RAUPACH and KLOEPPER, 1997). Summarized interactions between plant and growth–promoting bacteria are shown in figure 2.1. The bacteria locate plant roots through substances exuded from the root, and root exudates such as carbohydrates and amino acids stimulate growth–promoting bacteria (PGPB) chemotaxis on root surfaces (SOMERS *et al.,* 2004). Root exudates influence flagellar motility in some rhizospheric bacteria and make it possible to assume that microbial activity in the substrate is a function of root activity of the plants (DE WEERT *et al.,* 2002). Efflux of exudates in the root area of the plants on the one side as well as functioning microbial community on the other side conduce some PGPB to chemotaxis through flagella motility, creating positive association of microbes in substrates and reducing potential risk of root diseases (LUGTENBERG *et al.,* 2001).

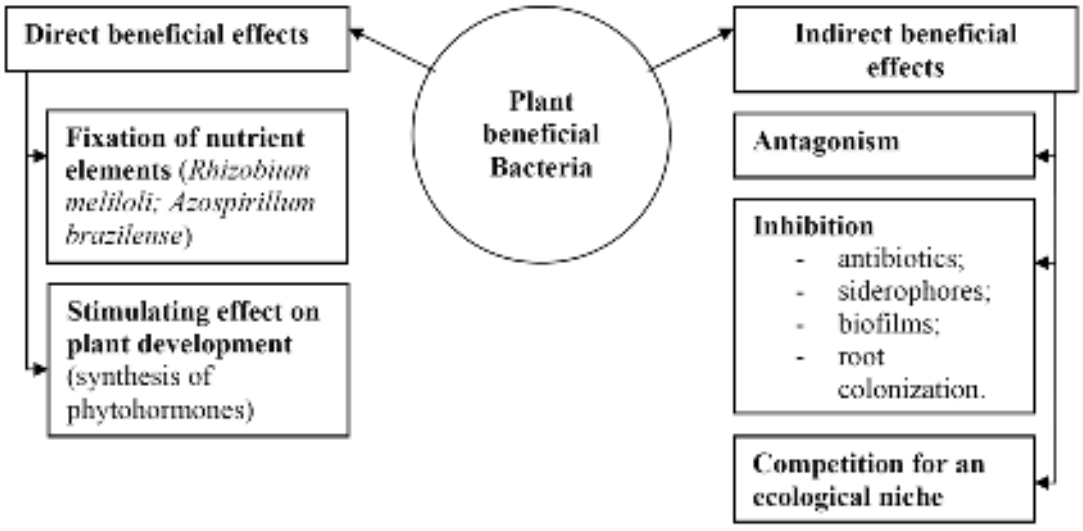


**Figure 2.1** Beneficial effects within plant-microorganism system (MADIGAN and MARTINKO, 2000)

Relative to wild-type bacteria, mutants had a strongly reduced ability to competitively colonize roots (DE WEERT *et al.,* 2002). Thus, chemotaxis appears to be important for competitive colonization by extracellular PGPB. The mechanisms responsible for this biocontrol activity include competition for nutrients, niche exclusion, induced systemic resistance (ISR), and the production of antifungal metabolites. The biocontrol agents that are best characterized at the molecular level belong to the genus *Pseudomonas.*

#### 2.2.1.1 Potential bacteria -*Bacillus subtilis*

*B. subtilis* (BS) is a common saprophytic inhabitant of soil, Gram-positive, rod-shaped, aerobic, and ubiquitous bacterium commonly found in soil, rotting plant material and is non-pathogenic (MADIGAN and MARTINKO, 2000). It is one of the most studied gram-positive bacteria. *B. subtilis* belongs to the *Genus Bacillus*, *Bacilaceae* family that belongs to *Bacillales* order which in turn subpositioned to *Bacilli* class, *Phylum Firmicutes* and united in the kingdom of bacteria. *B. subtilis* can be found in air, water but makes soil and organic substrates to its primary habitat. It has a physical size of 2.0-3.0 μm in length and 0.7-0.9 μm in diameter. One feature that has attracted a lot of interest in *B. subtilis* is its ability to differentiate and form endospores (SINCLAIR, 1989) that are highly tolerant to unfavorable conditions of local environment; it enables *B. subtilis* to withstand wide range of temperatures 5-55°C specifically heat and drought stresses (BAYLISS *et al.,* 1981; CLAUS and BERKELEY, 1986; SINCLAIR, 1989). *B. subtilis* does not possess traits that cause diseases and there is no evidence of its toxic effect on humans, animals, plants.

*B. subtilis* has a capacity to grow under a high range of temperatures; however, growth occurs normally under aerobic conditions with optimal temperature range of 24-27°C. There are evidences that in presence of nitrates it is capable to grow under anaerobic conditions, optimal pH values are 6.3-7 (CLAUS and BERKELEY, 1986). *B. subtilis* strain possesses a distinctive capacity to inhabit the root system of the plants after its application. The growth and multiplication of *B. subtilis* takes place on root system of treated plants, specifically on root hairs of the plant's root. Accretion of root hairs plays a crucial part in forming a suitable environment for BS establishment and development.

*B.subtilis* is a chemo-organo-heterotrophic microorganism which inhabits areas adjacent to rhizosphere as well as distributed through entire substrate's volume. In horticultural substrates, especially after long term usage, it is a common case when *B.subtilis* is distributed evenly through the substrate's volume. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this it not strictly correct (NAKANO and ZUBER, 1998).

#### 2.2.1.2 Function of *B.subtilis* as antagonist against diseases

The bacterium colonizes the developing root system of the plant and thus competes with certain fungal disease organisms (MAJUMDER *et al.,* 1985). *B. subtilis* is not considered a human pathogen; it may contaminate food but rarely causes food poisoning (RYAN *et al.,* 2004).

*Bacillus subtilis* is a bacterium that is used as a fungicide on flower and ornamental seeds, and on agricultural seeds including seeds for cotton, vegetables, peanuts, and soybeans. Several strains related to *B. subtilis* are used in the commercial production of extracellular enzymes, such as *B. amyloliquefaciens* alpha-amylase. Other strains produce insect toxins, peptide antibiotics and antifungal compounds, some of which have been used in agricultural crop protection. *B.subtilis* metabolizes a wide variety of carbon sources and secretes large quantities of industrially important enzymes (MUKHOPADHYAY *et al.,* 1985). Activity spectrum of *B. subtilis* is multipronged but most scientists (KILIAN *et al.,* 2000.) distinguish the ones described in figure 2.2.

Most common function of microorganism are:

1. Antibiosis;
2. Competition;
3. Induced resistance;
4. Growth promotion;
5. Yield increase;
6. Disease escape;
7. Improved plant strength.

***Antibiosis***. All forms of negative interaction between organisms that ranges from direct feudality to indirect impair of competing counterparts. Antibiosis is an antagonism mediated by specific or nonspecific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds or other toxic substances (JACKSON, 1965). *B. subtilis* is capable of producing specific compounds of antibacterial and antifungal nature (KATZ and DEMAIN, 1977). Compounds like *difficidin* and *oxydifficidin* have activity against a wide spectrum of aerobic and anaerobic bacteria (KIMURA and HIRANO, 1988). *Difficidin* and *oxydifficidin* are capable to reduce activity of microorganisms; at the same time *B. subtilis* possesses a capability to synthesize wide range of antibiotics such as *bacitracin*, *bacilin*, *bacillomycin B* (PARRY *et al.,* 1986; LOEFFLER *et al.,* 1986).

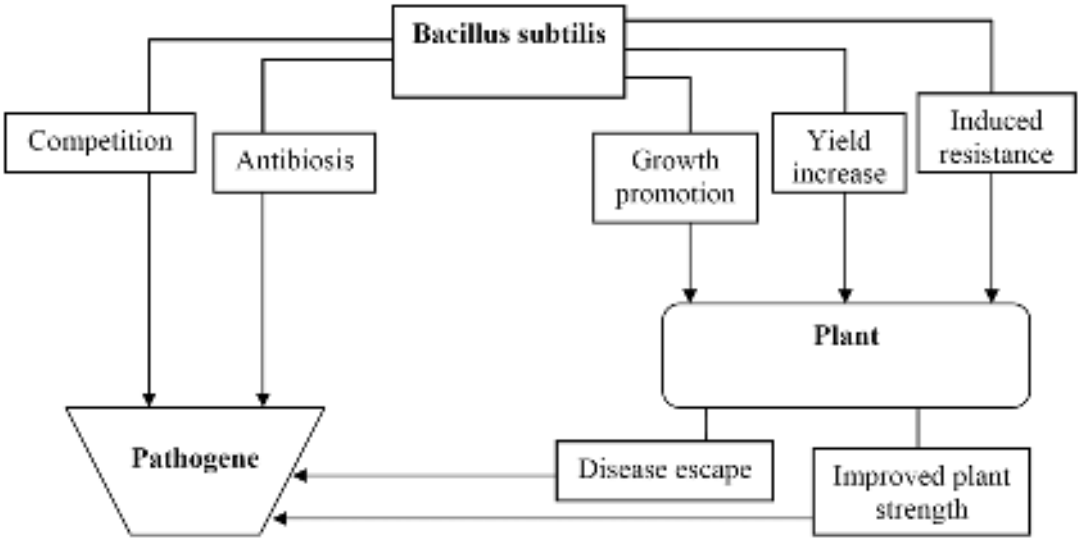


**Figure 2.2** Properties and effects of *Bacillus subtilis* (LOEFFLER *et al.,* 1986)

Application of *B. subtilis* leads to development of allelopathy which is a result of chemical compounds production under specific influences of biotic and abiotic factors. More, antibiotic entities have been described as products of strains of *Bacillus subtilis* than of any other species. Antibiotic activity is produced in a wide variety of media, including both inorganic and organic types. Different authors describe these antibiotics as follows:

***SUBTILIN***, first described by Jansen and Hirschmann (1944) shows some evidence of polypeptidic nature and is probably a complex substance of several factors. It has been studied by Salle & Jann (1946) who have shown it to be antagonistic chiefly to gram-positive bacteria. Subtilin as a product of *Bacillus subtilis* is antibiotic of peptide nature. Subtilin is synthesized via precursor proteins (NISHIO *et al.,* 1983; SHIBA *et al.,* 1985).

***BACITRACIN*** first described by Johnson *el al* (1945) is produced by a member of the *B. subtilis* group, and resembles *subtilin* in a number of ways, but differs from the latter by accumulating primarily in the culture liquor free from the cells.

A third agent of *B. subtilis* has been named ***BACILLIN*** (FOSTER and WOODRUFF, 1946). This substance is readily produced on media containing carbohydrate. It may be distinguished from *subtilin* and *bacitracin* by its high activity against both gram-positive and gram-negative bacteria in certain media.

The production of ***EUMYCIN*** by *B. subtilis* (Marburg strain) has been reported by Johnson and Burdon (1946). This substance without effect on gram-negative bacteria, inhibits staphylococci

only slightly, and shows considerable activity *in vitro* against *Corynebacteriurn helation, Mycobacterium tuberculosis,* and some of the higher pathogenic fungi.

Callow and D'Arcy Hart (1946) have recovered an antibiotic, subsequently termed ***LICHENIFORMIN***, from the cells of *Bacillus licheniformis*. According to Saris *et al.,* (1990) the responsible organism is probably identical with *Bacillus subtilis* (Ford strain). The active agent has little or no effect on gram-negative microorganisms, but inhibits certain gram-positive. Among these are ***SUBTILYSIN*** (*SUBTILYNE*) (VALLYE, 1945) which is strongly toxic towards certain gram-negative and gram-positive organisms.

The antibiotic called ***ENDOSUBTILYSIN*** discovered by de Saint-Rat & Olivier (1946) is reported to have nontoxic and in high dilution, can be bactericidal for staphylococci. Ramon and Richou (1946) have reported the formation of a substance called *SUBTILINE* which inactivates certain bacteria *in vitro*. ***SUBTILOSIN A***, has been found in *Bacillus subtilis* (SPECTOR, 1982., BABASAKI *et al.,* 1985). It has suppressing influence on certain gram-negative and gram-positive organisms.

***MYCOBACILLIN.*** *B. subtilis* was first time described by Ghosh *et al.,* (1983) and stated to have toxic effects against both gram-positive and gram-negative microorganisms. All these antibiotics that are being synthesized under certain environmental conditions and influence development of microbial community in the root area of the plants, define functions of *B. subtilis* that make it useful as a plant strengthener.

***Competition***. Development of the root system of the plant is accompanied by the variety of metabolite processes which result in secretion of exudates and discardment of epidermis cells into intermediate environment. In fact, according to **(**GRIFFIN *et al*., 1976**)** these exudates account for 98% of all carbohydrate material that is being released by plants and have chemotactic effects on microorganisms and stimulate their sporulation and growth. The next stage of energy and material flow in biosphere in this case is decomposition of organic matter by different microorganisms lead to the situation where microflora is actually forced to compete for those nutrients being discarded by plants. Thus, ability of certain microorganism to reproduce itself leads to increased density of microbial community per volume of rhizosphere space, which in turn, leads to higher competition for area around plant root system. *B. subtilis* is able to take up to the plants root only in presence of a thin film of water on the root hairs. In this case root exudates are used as a nutritious substrate for supporting its own metabolism. Critical factors for growth and development of *B. subtilis* colonies are availability of water or in case of soilless

culture substrate water holding capacity, organic matter content in the substrate and frequency of nutrient solution supply (NAKANO and ZUBER, 1998). Average concentration of *bacilli* in soil is $10^6$ to $10^7$ cfu per gram of soil and most of it in the in the active spore state (90-100%). However amendment of soils through addition of organic matter causes exponential growth of the bacilli colonies (ALEXANDER, 1977). Relationship between organic matter content and microbiological activity was shown in pot experiments with maize in various soils with different organic matter content (ZIMMER *et al.*, 1998). In experiments with *B. subtilis* FZB24® and its population development on maize roots and in the soil after seed treatment was shown that the number of spores and cells tend to increase on variants with higher organic matter content (KILIAN *et al.,* 2000.)

***Induced resistance***. Many agricultural plants in their ontogenesis can produce a variety of substances called *pathogenesis related* proteins which are perceived as markers of induced resistance (FOSSUM *et al.,* 1986). Bacteria in their interactions with plants produce a number of metabolites that are thought to be the triggers of induced resistance in higher plants against pathogens. These compounds include lipopolysaccharides (KLIER *et al.,* 1983), enzymes and siderophores (LEEMAN *et al.,* 1995), salicylic acid (MEYER *et al.,* 1997). In experiments with different plants infected with fungal pathogens, application of *B. subtilis FZB24*® to the root system of tomatoes showed decrease in *Phytophthora infestans* and by *Botrytis cinerea.* Biologically active substances as metabolites of *B. subtilis FZB24*® activity often trigger induced resistance against malicious microorganisms (*F.oxysporum and Lycopersicon esculentum*). Plants treated with *B.subtilis* showed positive results in comparison to control plants without treatment (DOLEJ and BOCHOW, 1996).

Metabolic processes within *B.subtilis* cells induce synthesis of different antibiotics and proteins as well as protein complexes (BOCHOW, 1998). Synthesis of protease, alpha-amylase and lipase by *B.subtilis* gains on its intensity upon its interaction with the plant root system and its exudates and plays an important role in inducing plant's resistance to malicious microorganisms (FZB Biotechnik GmbH, 1995).

#### 2.2.1.3 Function as growth stimulator

*Bacillus subtilis* FZB24 WG is the only strain that was used in the experimental part of this work. This strain is commercially available and it has been produced by FZB Biotechnik GmbH. *Bacillus subtilis* FZB 24® is registered under the number **Nr. LS 004954-00-00** by (**Biologischer Bundesanstalt für Land- und Forstwirtschaft**) of plant strengthening substances. That fact

serves as a legal basis for application and utilization of given product in agricultural research and production purposes in Germany.

***Growth promotion***. Introduction of *B. subtilis* into the rhizosphere through colonization of the roots and rhezoplane by *B. subtilis FZB24*® contributes to growth promotion of the plants. The mechanism of root recognition on bacterial side connected to major outer membrane protein (MOMP) (LUGTENBERG *et al.,* 2001). As an example of MOMPs from *Azospirillum brasilense* they are capable to bind to membrane-immobilized root extracts from several plant species with differing affinities. Some plant growth promoting bacteria (PGPB) produce phytostimulators, which directly enhance plant growth. There are evidences that PGPBs are capable of the atmospheric nitrogen fixation and *Azospirillum* spp. secretes phytohormones such as auxins, cytokinins, and gibberellins (STEENHOUDT *et al.,* 2000). Bacteria are capable of producing growth regulators continuously, provided that precursors of phytohormones are available in the rhizosphere. The root exudates can supply the range of precursors that are capable to induce a biotransformation of PGPBs (JAEGER *et al*., 1999). The study showed the availability of tryptophan mainly near the root tip region. Tryptophan is the precursor for a major auxin, indole-3-acetic acid (COOKE *et al.,* 2002), suggesting that PGPB could exploit root exudates pools for various precursors of growth regulators. Other rhizobacteria create "suppressive soils" by controlling plant diseases caused by soil fungi and bacteria.

***Yield increase.*** Microbiological activity as a function of abundant nutrients availability in the rhizosphere and some of these rhizobacteria provide benefits to the plant, resulting in plant growth stimulation (GRAY and SMITH, 2005). An application of *B. subtilis* can reduce concentration of malicious substances in the substrate through its capacity. Microbial complexing agents can be the low molecular weight organic acids and alcohols, the high molecular weight ligands, siderophores, and toxic metal binding compounds. All these agents can lead to increase in plants productivity as well as improvement of yield quality. The use of chelation agents may be useful in mobilizing toxic inorganic compounds to facilitate their removal from solid waste (MALCOLM and VAUGHAN, 1979). Some amino acids formed by bacteria can also be complexing agents. The complexation mechanism is common for any substance that is capable to bind anions in compounds is illustrated as follows:

**Metal + Ligand => Metal complex**

Ligands in these case are low molecular weight compounds as various organic acids (citric acid, tricarboxylic acids) released during microbial degradation had been found to have metal chelation ability. The rank order of the complexing ability of organic acids:

**Tricarboxylic acids > dicarboxylic acids > monocarboxylic acids**

Dolej and Bochow (1996), Krebs *et al.,* (1998) claim that application of *B. subtilis* FZB24® brings positive effects on plants growth and yield increase as a result of root colonization. The yield and growth increase in variants with application of *B. subtilis* can be explained by the fact that compounds synthesized by bacilli as well as other substances such as peptides; proteins can interact with plants causing biostimulating effects (LOGAN and BERKELEY, 1981; DOLEJ and BOCHOW, 1996; BOCHOW *et al.,* 1999). *In vitro* cultures of *B.subtilis* manifest presence of antibiotic-like substances (BROADBENT *et al.,* 1977), cytokinins-like effects (STEINER, 1990; ZASPEL, 1992). The presence of *B.subtilis* positively influenced growth promotion of *Pinus pinea* by developing auxin- and cytokinins-like substances what resulted in better root and shoot growth (O'DONNELL *et al.,* 1980).

***Disease escape.*** *B. subtilis* FZB24® forms mainly serine-specific endopeptidases that can be transported outside the bacterial cell, inhibiting other microorganisms (PRIEST *et al.,* 1982). Most effective way of disease escape is a constant production of antibiotics that suppress development of plant's diseases as well as competition of *B. subtilis* FZB24® in root area of the plant (SARIS *et al.*, 1989; TATTINI *et al.,* 1989). Creation of sufficient conditions (aerobic conditions, water availability in substrate) for growth of *B.subtilis* provides sustainable disease escape effect.

***Improved plant strength***

*In situ* the resistance systemically induced in tobacco by extracellular pectinases and cellulases of *Erwinia carotovora* is probably due to the release of cell wall fragments as signals for the activation of defense genes (PALVA, 1990). *B.subtilis* contributes to increased plant growth through different mechanisms, like suppression of malicious microorganisms as well as production of different bioactive compounds that influence plant's root development which in turn takes up more nutrient elements (GORDON, 1983). Increase in biomass production of horticultural crops through application of *B. subtilis* triggered through increase of root biomass

and subsequently green biomass of the plants provides prerequisites for tolerance against different suboptimal biotic and abiotic growing factors and improved overall plat performance (KOCH, 1996; ZIMMER *et al.,* 1999).

**Suboptimal growing conditions**

*Bacillus subtilis* FZB24® is credited with an ability to evolve different kinds of stress protective mechanisms including stimulation of plants own defense mechanisms. Introduction of *B.subtilis* in vegetation system with suboptimal abiotic factors proved to have positive aggregate effect on the test plants (BOCHOW *et al.,* 2001; OBI, 1980). *B. subtilis* FZB24® proved to be efficient in reliving of different kinds of stress factors such as suboptimal pH, EC, temperature (BECKERING *et al.,* 2002). *B. subtilis* FZB24® responds to a drastic fluctuation of temperature through so called heat shock response. This specific reaction paves the way to the production of shock proteins, which allow the cell to cope with the stress regimes (SCHUMANN, 2003). Biotic stresses can be also countered by application of *B. subtilis* FZB24®. Positive results can be achieved through stimulation of plant growth combined application of biostimulating agents designed for the containment of negative effects attributed to suboptimal growth factors (MURPHY *et al.*, 1999). Experiments with different values of EC and *B. subtilis* treatment proved that microorganism is capable of salinity stress reduction (WOITKE *et al.,* 2004) As a result, *B. subtilis* is reported to increase leaf area of tomato plants under conditions of salinity at the same time having less or no effect on salinity itself. Comparison between variants treated with BS (0.05% w/w; 7-times; 50 ml/plant) and relationship of dry weight/fresh weight indicates that presence of *B. subtilis* contributed to increased water content in plant leaves. Variants with other treatments lead to conclusion of stress-reducing effect on inoculated plants. Variants with high EC values and with application of *B. subtilis* and without it had 20% lower yield of tomato fruits. More than 90% of deficient fruits had symptoms of blossom end rot (BER). The fruit set value was on decreasing trend on all variants except control and was lowest on the variant with *B. subtilis* treatment. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. *B. Subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. However, under most conditions the organism is not biologically active but exists in the spore form (ALEXANDER, 1977). Saprophytic lifestyle of *Bacillus subtilis* contributes to mineralization and mobilization of organic compounds back to geochemical cycle. Microorganism has a variety of glucan- and protein degrading enzymes that can be exported

from the cell. As long as there is an ample pool of nutrient elements *B.subtilis* colonies that are dull and may be wrinkled, cream to brown in color and when grown in broth have a coherent pellicle; usually with a single arrangement. Like most members of the genus, *B. subtilis* is aerobic, except in the presence of glucose and nitrate, some anaerobic growth can occur (CLAUS *et al*., 1986).

#### 2.2.1.4 Description of other microorganisms

Economically important diseases of greenhouse crops are damping off, root rot, stem rots, *Fusarium, Verticillium* wilt. Application of chemical agents for the pest and disease control cause systemic instability in greenhouses; some active substances are hazardous to human health and the environment. Among different possibilities to control soil-borne diseases and pests in greenhouses, biological control is one of the decisions in modern plant protection (DORMANNS-SIMON, 1995). Establishing the composition of antagonistic microorganisms towards substrate-borne phytopathogens is especially important from the point of view of biological protection of plants. Introduction of antagonistic microorganisms limiting the occurrence of pathogenic substrate-borne fungi paves the way to development of biologic control strategies in horticultural practices (AHMED *et al.,* 2000). A huge role in limiting the occurrence of pathogenic fungi in the substrate is played by antagonistic bacteria *Pseudomonas spp*. (AHMED *et al.,* 2000) as well as by fungi *Gliocladium spp*. (KREDICS *et al.,* 2000) and *Trichoderma spp*. (McQUILKEN *et al.,* 2001). Biological agents are much more sensitive to different conditions than chemicals. Soil pH, aerobe or anaerobe circumstances, availability of certain nutrients, temperature, humidity, all have an effect and may substantially determine the efficacy and the persistence of the biopreparate. For instance, the effect of the fungal biocontrol agent *Gliocladium virens* in experiments with cucumber plants showed that both damping off and pathogen population were significantly reduced. Application of biological agents has its downside - efficacy of biological agents never reaches 100%. The main task of such biopreparates is to decrease the damage below certain threshold – but they should not change the soil microflora significantly – as chemical pesticides do (DORMANNS-SIMON, 1995).

### 2.2.2 Use of humates in horticulture

**Humus** – labile, unstructureralized compound of bioactive substances resulted from deterioration of primarily plant material. Humus is a valuable substance of soil and agricultural substrates that influences their chemical and physical properties and increases their sustainability

in natural and commercial utilization. The components of humus possess different molecular sizes and their molecular weights fall into range between 300 (fraction of fulvic acids) to 300.000 atomic mass units (Mc CARTHY *et al.,* 1990). Humus itself is a complex mixture of different compounds by physical as well as chemical properties. These substances are often referred to as humic substances and are classified according to their solubility in different solvents (FLAIG, 1966). Humates are salts of humic acids with different chemical and physical properties. Humates can be also represented as combined components of fulvic, humic acids and humin (Mc CARTHY *et al.,* 1990). It is common to refer to humates as humic substances. From 20 to 70% of the exchange capacity of many soils is caused by colloidal humic substances. As far as buffer action is concerned, humus exhibits buffering over a wide pH range. Total acidities of isolated fractions of humus range from 300 to 1400 meq $100g^{-1}$ (CHEN *et al.,* 1977).

#### 2.2.2.1 Classification and sources of humates

Humate materials are widely distributed organic carbon containing compounds found in soils, fresh water, and oceans. Humic substances are involved in the decomposition of rocks and minerals. The decomposition of various minerals by solutions of humic acids has been demonstrated by many investigators (DIAZ-BURGOS *et al.,* 1993; GAUR and MATHER, 1996; GOVINDASMY and CHANDRASEKARAN 1992). The character of the action depends on the nature of the humic substances, and on the resistance of the minerals. However, the chemistry and function of the organic matter has been a subject of controversy since beginning of their postulating in the 18th century. Until the time of *Liebig*, it was supposed that humus was used directly by plants, but, after *Liebig* had shown that plant growth depended upon inorganic compounds, many soil scientists held the view that organic matter was useful for fertility only as it was broken down with the release of its constituent nutrient elements into inorganic forms (HAJRA and DEBNATH, 1985).

**Humic acid** is ubiquitously present complex mixture of organic biopolymers resulted from decomposition processes on incoming organic material such as remnants of plant and animal materials. Humic acids are complex polymers which include amino acids, amino sugars, peptides, aliphatic compounds involved in linkages between the aromatic groups (FRIMMEL and CHRISTMAN, 1988). Complexity of the humic acids underlines their different physical and chemical properties. The hypothetical structure of humic compound contains free and bound phenolic–OH groups, nitrogen and oxygen as bridge units and –COOH groups variously placed on aromatic rings (Figure 2.3).

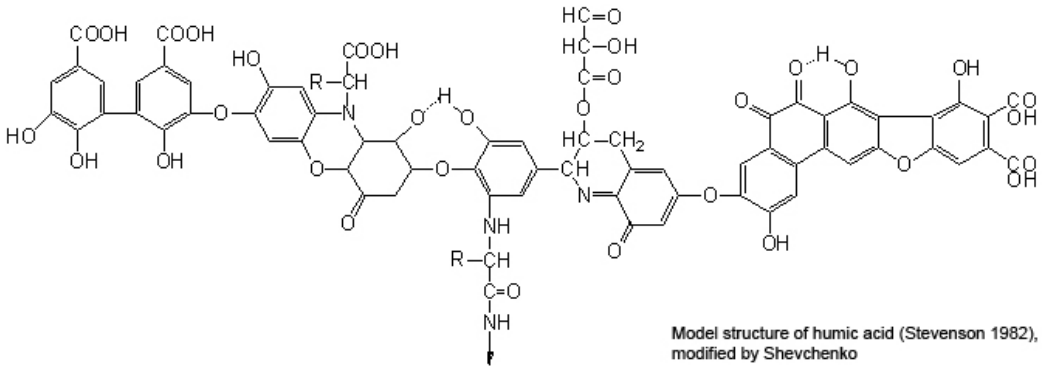


**Figure 2.3** Fragment of humic acid chain

Humic substances represent a group of organic residue of decaying organic matter. Organic compound, are any compound of carbon and another element or a radical (Figure 2.4).

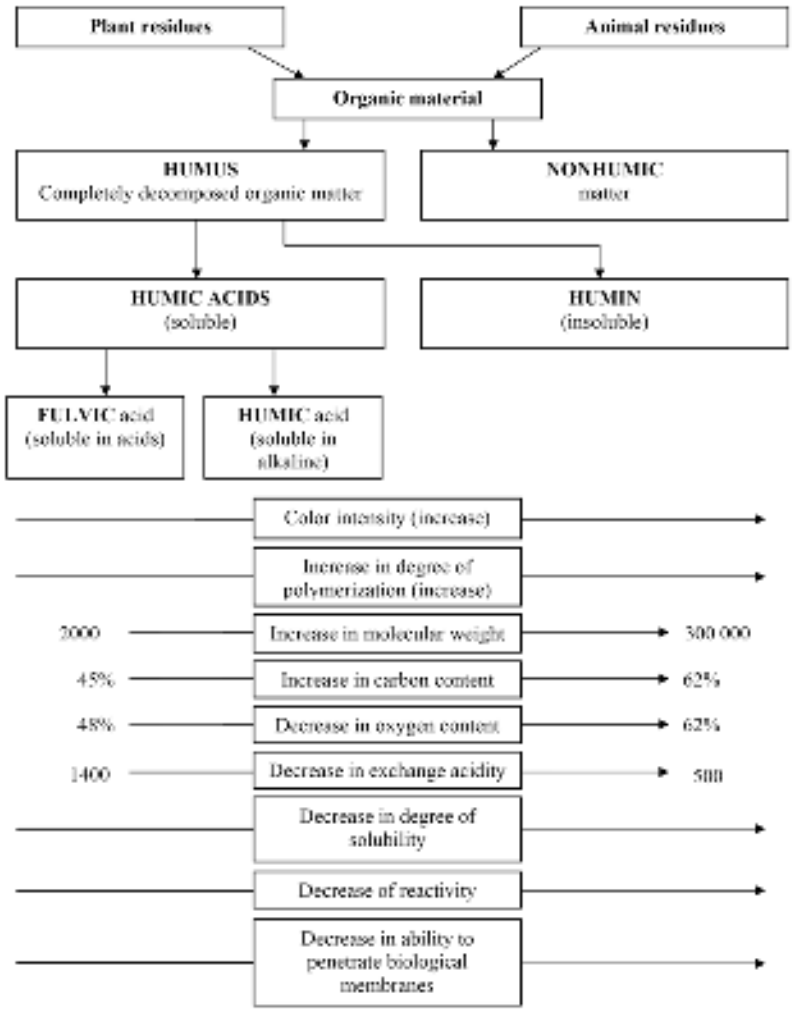


**Figure 2.4** Origin and chemical properties of humic substances (STEVENSON, 1982)

Humic substances exhibit different characteristics as to solubility in water or other solutes and pigmentation. With regard to their physical and chemical properties, humic substances are classified as follows:

**fulvic acid** – a yellow to yellow-brown humic substance that is soluble in water under all pH conditions; "they measured the fluvial fulvic acid"

**humic acid** – a dark brown humic substance that is soluble in water only at pH values greater than 2; "the half-life of humic acid is measured in centuries"

**humin** – a black humic substance that is not soluble in water.

Further classification of humic compounds is formed on different chemical and physical properties such as color, solubility in different mediums, molecular weight, oxygen content, and degree of exchanged acidity. Humic acid (HA) are macromolecules and generally referred to as humates. These macromolecules are the substances of very complex structure (its molecular mass is 160000 atomic mass units and can vary in great measures) and practically insoluble in water, except for a very small part called fulfonic acids. Their structures have not yet been fully characterized, although certain functional groups, such as carboxyl, alcohol, and phenol are common to all humic macromolecules.

Recent studies suggest that HAs are very complex mixture of different compounds like sugars, organic acids and many other substances of aliphatic and aromatic nature (PICCOLO *et al.,* 2002). From ecological point of view, these macromolecules plays on soils and substrates contaminated with different xenobiotics (heavy metals, radionuclides). Possessing such a sorptive capacity HA can prevent or drastically decrease income of pollutants into the trophic cycles. These macromolecular structures have a role to play in bioremediation of organic pollutants like metabolites and rests of pesticides (HOANG, 1996).

To have it soluble, $H^+$ in humic acid molecule, must be exchanged for alkali metals $Na^+$ or for that matter $K^+$. This chemical substitution increases biological activity of humic compounds and increases the potential for their use in horticulture (formula 1).

$$\text{HUMIC ACID} + \text{KOH} = \text{HUMATE-K} + H_2O \qquad (1)$$

The formula above shows how, as a result of this treatment, hydrogen atoms in carboxyl and hydroxyl groups are replaced by alkali-metal ions. The reaction with KOH leads to dissociation, which results in acquiring a charge by molecule of humate. Distribution of these charges along the molecule length leads to repulsion between different parts of the molecule. The humate molecule stretches out. It allows the humic acid molecules to pass into solution and to become

biologically active. Functional groups of humic acid play their certain roles and influence plant on different stages of growth. For example, carboxyl and phenol groups are able to form chelate complexes with microelements and transport them into plants in this form (CANTOR and SCHIMMEL, 1980).

**Cation-exchange capacity.** Colloidal nature of humic acids underlies their capacity to attract, bind, hold and exchange anions and cations (MATHUR and FARNHAM, 1985). Because of the high molecular weight, the negative surplus charge on their surfaces is insufficient for peptizing the macromolecules even at strongly alkaline pH, which implies that that mobility of these substances can be very significant once in coagulated state (EVANGELOU, 1998). Cation-exchange capacity (CEC) of HAs is a very important property that can play a decisive role especially in combination with such substrates like rockwool and perlite. In terms of physical properties, humic substances have a tremendous surface area. Together with the high number of exchangeable $H^+$ ions can significantly increase the CEC. A substantial fraction of the mass of the humic acids is in carboxylic acid functional groups, which endow these molecules with the ability to chelate multivalent cations such as $Ca^{2+}$, $Mg^{2+}$, S, $Fe^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Fe^{3+}$, and $Mo^{2+}$ (SCHNITZER *et al.,* 1967; WEBER, 1988). Some substrates, mainly of inorganic origin do not possess significant cation-exchange capacity, and thus, buffering capacity. The cation-exchange capacity (CEC) of commercially produced humic acid is in the range of 500 to 600 meq 100 $g^{-1}$ (CANTOR *et al.,* 1980). This is about five times greater than the CEC of good quality peat moss and twice as high as the CEC of soil humus (CANTOR and SCHIMMEL, 1980).

**Chelating agents.** Chelation is a process which is conditioned by particular physical and chemical characteristics of curtain compounds (SWIFT, 1996). There are varieties of substances, including humic compounds which are known as biopolymers and have strong chelating capacity with regard to nutrient ions. Humates are capable of retaining some of the elements on the specific sites of their molecules and deliver them back into the solution once conditions are changed (SWIFT, 1996). The soilless culture for vegetable production has increased demands for nutrient elements because of the nutrients mobility within the system. The major task for achieving a sustainable nutrient supply in the soilless culture is not confined just to delivery of the nutrient solution to plants but also extends to retention of already available nutrients from precipitation or leaching from the substrate. Transport of different nutrient elements can be facilitated through chelating process. Nutrient elements (carbon, hydrogen, nitrogen, calcium, phosphorous, sodium, potassium, sulphur, and magnesium) as part of complex (chelated)

compound are more resistant to leaching and weathering processes and available for variety of metabolic processes (SCHNITZER and SKINNER, 1964). Chelate with metal ions is formed when two or more coordinate positions about the metal ion are occupied by donor groups of a single ligand to form an internal ring structure. In soil, fulfillment of ligand role belongs to simple organic compounds and functional groups of humic substances (PATERSON *et al.,* 1991). The order of decreasing affinity of organic groupings for metal ions can be presented as follows:

$$\text{-O- > -NH}_2\text{ > -N=N- > =N > -COO- > -O- > C=O}$$

The chelating property of K-Humate comes from its chemical constitution that contains an array of functional groups, such as –COOH, phenolic, -OH and =C=O groups. Soil organic constituents form both soluble and insoluble complexes with metal ions and thereby play a dual role in soil. Low – molecular – weight compounds (fulvic acids) bring about the chelation of metal ions and affect their transport to plant roots. The order of decreasing ability of metal ions to chelating is as follows:

$$Fe^{3+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Fe^{2+} > Mn^{2+}$$

HAs with higher molecular weight possesses a capacity to bind polyvalent cations (GECKEIS *et al.,* 2002). Being introduced to nutrient solution, such micronutrients like $Fe^{2+}$, $Fe^{3+}$, $Mn^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Cu^{2+}$ do not become accessible to plant instantly. The reason for this is their partial insolubility in case of being provided as common inorganic salts. In the substrate, however, the alkaline cations ($Na^{+}$, $K^{+}$, $Ca^{2+}$, $Mg^{2+}$) are held primarily by simple cation exchange with –COOH groups (RCOO-Na, RCOO-K etc.) (YONEBAYASHI and HATTORI, 1988). The humates and fulvates occur in the soil largely as mixture with hydroxide of $Fe^{2+}$ and $Al^{3+}$. This ability of some substances to form coordinate bonds to metal ions is called chelation. In most cases the coordinate bond is formed through oxygen and/or nitrogen donor atoms. It is also known that most of the substances capable to form coordinate bonds to a metal-ion, are of organic nature (PLASCHKE and FANGHÄNEL, 2004). An ability to act as a chelating agent is the most important interaction between humic acids and ions available in nutrient solution. Creating complex compounds with nutrient elements, humic substances increase their uptake in two ways:

- preventing ions from precipitation through forming fully chelated compounds;
- increasing bioavailability of nutrient elements in Substrate-Plant-Microorganism system.

### 2.2.2.2 Effects of humates on plant growth

**Direct effects of potassium humate.** Effects from application of K-Humate can be divided into two groups – direct that are observed on the plants and indirect effects in the substrate as described in figure 2.5.

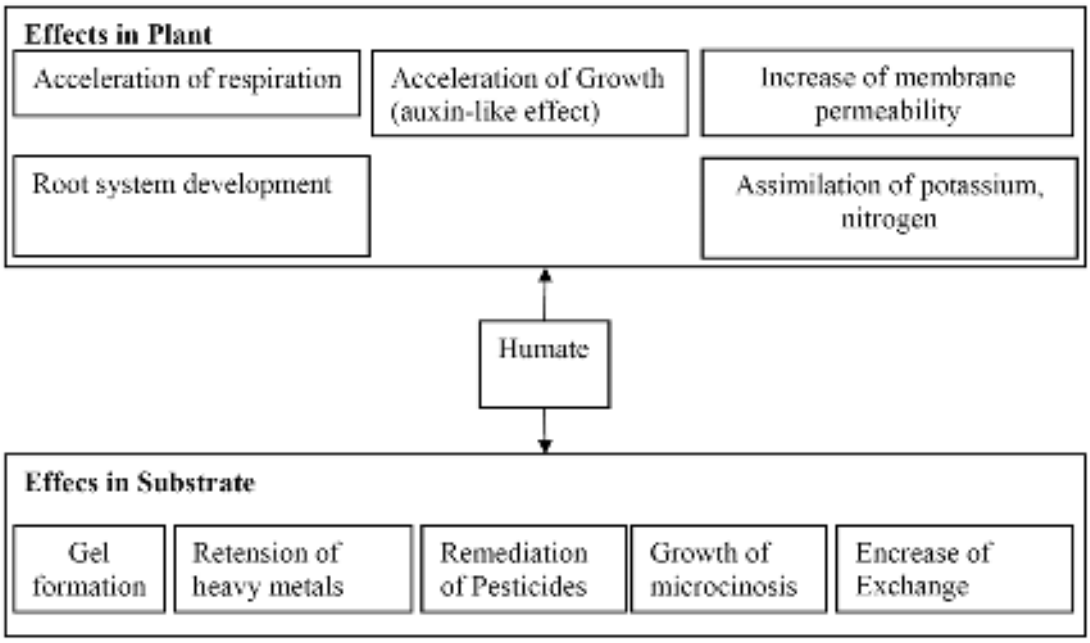


**Figure 2.5** Effects of humates on plants and substrates

Intensive agricultural systems demand the use of large quantities of mineral fertilizers in order to supply the plants with basic macro-elements, such as nitrogen, phosphorus, and potassium. Application of these fertilizers in the pure form as well as in the nutrient solution in horticulture can lead to partial loss of these nutrients. Phosphorus fertilizers, on the contrary, react with cations of $Ca^{2+}$, $Mg^{2+}$, $Al^{3+}$, and $Fe^{3+}$ that are present in soil or substrate and form inert compounds. These inert compounds are either partially or completely in accessible for plant's root system. The presence of humic substances substantially increases the effective assimilation of all mineral nutrition elements. It was shown in a test with barley that humate treatment (with NPK) improved its growth, development, and the crop capacity while decreasing the use of mineral fertilizer (BORTIATYNSKI *et al.,* 1996.). Soil phosphates are often immobilized through reactions with iron and aluminum, which in turn creates complexed compounds with organic matter. Chelating agents can break the iron or aluminum bonds between the phosphate and organic matter, releasing phosphate ions into solution. This dissolution is a process which occurs in soil in the presence of naturally-occurring humic substances or plant root exudates. The

addition of humates may increase the rate of this process, thereby increasing the availability of phosphorus to plants.

**Development of the root system**. Humic acids can have a direct positive effect on plant growth in a number of ways. They have been shown to stimulate seed germination of several varieties of crops (CHEN and AVIAD, 1990). Both plant root and top growth have been stimulated by humates, but the effect is usually more prominent in the roots. A proliferation in root growth, resulting in an increased efficiency of the root system, is a likely cause of higher plant yields seen in response to humic acid treatment (CLAPP *et al.,* 2001). Humic matter has been shown to increase the uptake of nitrogen by plants, and to increase soil nitrogen utilization efficiency. It also enhances the uptake of potassium, calcium, magnesium and phosphorus. Chlorosis in plants has been prevented or corrected by humate application, probably the result of the ability of humate to hold soil iron in a form which can be assimilated (CHEN and AVIAD, 1990). This phenomenon can be particularly effective in alkaline, calcareous soils, which are normally deficient in available iron and low in organic matter content.

**Increase of membrane permeability**. Among the effects conduced by application of humic acids on plants is the increase in penetrability of the cell membrane of the leaves, effects aggregate productivity of entire plant (SENN and KINGMAN, 1973.). Salts of humic acids increase permeability of cell membranes; they also increase efficiency of enzymes responsible for breathing and synthesis of proteins and sugars. It facilitates the respiration of the plants (NARDI *et al.,* 2002). Increase in penetrability of the cell membrane facilitates the penetration of nutrients into the cell and accelerates the respiration of the plants. The humic acid acts as dilator increasing the cell wall permeability. This increased permeability allows easier transfer of cations (SENN and KINGMAN, 1973).

**Acceleration of respiration.** Application of humic acids in the root area of the plants stimulates and improves plant's nourishment. It facilitates uptake of nutrient element by the root system of the plants. Increased transport of ions as a function of humic acid application has selective nature. For example, the penetration of potassium ions increases a hundred times while sodium penetration increases ten times, this favorably influences plants' nourishment. (MUSCOLO *et al.,* 1999).

**Assimilation of potassium and nitrogen.** An experiment with winter wheat showed that one-way use of nitrogen fertilizers on winter wheat crops did not have a high positive effect on the crop capacity, while its use along with humates and super phosphate achieved an expected positive effect (STEVENSON, 1994). Interestingly, the mechanism of interaction between

humates and macro-elements of mineral nutrition is specific for each of them (GECKEIS *et al.,* 2002). The positive process of nitrogen assimilation occurs due to an intensification of the ion-exchange processes, while the negative process of "nitrate" formation decelerates. Potassium assimilation accelerates due to a selective increase in the penetrability of cell membranes. As for phosphorus, humates tend to bond ions of $Ca^{2+}$, $Mg^{2+}$, and $Al^{3+}$ first, which prevents the formation of insoluble phosphates. That is why the increase of humate content leads to an increase of the plant's phosphorus consumption (AVERETT *et al.,* 1995). It is important to point out that this process is rather selective. For example, the penetration of potassium ions increases a hundred times while sodium penetration increases ten times.

**Indirect effects of potassium humate**

**Gel formation** has contributed to the creation of structuralized substrate, activation of microflora and influence turnover of nutrient in the substrate (BORTIATYNSKI *et al.,* 1996). The capacity to form gel formations is attributed to the fact that humic substances that interacts with liquids start to "roll up" their complex structure and different functional groups capable to retain this liquids in close proximity (AVERETT *et al.,* 1995).

**Retention of heavy metals.** Heavy metals (HM) can form strong complexes with both inorganic and organic contaminants and mineral surfaces, and thus play a major role in geochemical processes (STEVENSON, 1994.). Bonding capacity of humic acids can be very efficient in heavy metal retention in the substrates. On the territories contaminated by heavy metals and radionuclides it was proved that uptake of these xenobiotics on the variants with application of humates is lower than on control variants.

**Remediation of Pesticides.** Pest and disease control involve application of toxic compounds that produce a variety of metabolites. Previews experiments show that application of humates can contribute to degradation of malicious metabolites of pesticides in the soil (NARDI *et al.,* 2002).

**Growth of microbial community**. The ability of the microbial community of the substrate to improve uptake of nutrient elements by the plants humates contribute to enhanced root development and root activity (exudates). The developed root system improves microbiological activity in the substrate and particular – root zone (MALCOLM and VAUGHAN, 1979).

### 2.2.3 Use of lactates in horticulture

Regulation of chemo-physiological processes within a plant pose a requirement on a variety of nutrient elements that must be available for plant's optimal growth and development as well as

its sufficient productivity. Application of lactates can lead to the rapid delivery of the main nutrients to the plants. Foliar application of the lactates facilitates improvement in plant nutrition. The four main nutrients for the plant are nitrogen (N), phosphorus (P) and potassium (K), calcium (Ca) can be present in different forms and therefore have a different availability for the root system of the plant. The microelements or otherwise known as trace elements are: manganese (Mn), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo), iron (Fe). Application of lactates by the watering into the rhizosphere of the plants can regulate of enzymatic activity in substrate, microbial community as well as in the plants. Availability of the nutrient delivered by the lactates for the plant nutrition can play stress relieving role under the suboptimal growing conditions.

#### 2.2.3.1 Description of lactates

Lactates, salts of lactic acid, can be used to chelate nutrients, especially micronutrients. Stress reducing effects of lactates can be found especially in nutrient solutions with too low or too high pH values and also in stress situations because of extreme temperature (BOEHME *et al.,* 2000). Several products of a Bulgarian company ECOFOL are offered as foliar fertilizer with the brand name LACTOFOL.

Lactofol® is a suspension fertilizer for leaf nutrition of plants. The suspension fertilizer "Lactofol" was developed for leaf nutrition of agricultural plants. Its use results in an average 8 to 10% increase in the yields of wheat, and in an increase in protein content of the grains of 0.5 to 1%. The suspension fertilizer consists of a biotechnological product (liquid phase) and a solid phase comprising nutrient macro- and micro-elements. The presence of protein hydrolyzate (amino acids) in the fertilizer is yet another major advantage: assimilated by the plant, it joins its metabolism and accumulates in grains in the form of protein. Lactofol"O"® contains no filler chlorine ions and can be used in large doses without any harmful effect on plants. This constitutes a major advantage for small-scale application. The good consistency of the fertilizers makes its spraying possible by means of fine ejection equipment. The production of LACTOFOL"O"® is environmentally safe as the raw materials used for its production consist of waste material from the dairy industry. The fertilizer is absorbed by agricultural plants together with pesticides. The following inventors are part of the invention team: Mr. Kostadin K. Kostadinov, Mr. Plamen I. Trifonov, Mr. Pavel Z. Bachvarov and Mr. Evgeni S. Ivanichkov. The final product is obtained through biotechnological processing of different products containing lactose and microelements. Lactic acid due to its chemical properties is capable of creating

complex compounds with mineral cations of nutrient elements. Lactic acid builds a dynamic balance of nutrient elements.

Lactic acid is known to be strong chelating agent and is capable to bind a variety of nutrient elements in form of cations in its coordination area. Reversibility of chelating bounds makes it very useful in leaf application and allows to support plants with nutrient elements in the critical stage of their development. Upon application of Lactofol® on leaf surface, chelated cations of Lactate travel against concentration gradient from its coordination area into leaf apparatus and enter metabolism processes. Leaf application of Lactofol is helpful in dealing with micronutrient deficiency during vegetation period of horticultural plants (RANKOV, 1992). Lactofol® contains ions of metals like $Fe^{3+}$, $Zn^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Mo^{2+}$ positively influence the photosynthetic activity of the plants. From experiments with sunflower it is also known that application of nutrient suspense results 15% increase in oil content (PAVLOVA, 2002).

Lactofol"O"® - is a suspension of micro- and macro- elements in concentrated form. It contains nitrogen in form of ammonium ($NH^+_4$), nitrate ($N\text{-}NO_3^-$) and amide ($R\text{-}CONH_2$). Upon application of Lactofol these forms of nitrogen enter and increase metabolism in the plant (RANKOV, 1992). Increase of metabolism can be expected due to availability of $NH^+_4$ in Lactate. Incorporation of nitrogen in form of ammonium takes much less time whilst it is in reduced form in comparison to nitrate. The problem here is that absorbed nitrogen is useless unless it is incorporated into organic molecules. Incorporation of nitrogen, especially in the form of nitrate, generally occurs in the root system of plants and requires availability of carbohydrates – products of photosynthesis. Thus, application of Lactofol as leaf fertilizer can lead to accumulation of nitrates in plant's tissues and potentially reduce quality parameters of the final product. Creation of optimal nutritional conditions in the substrate is very critical from the very onset of plants growth (RANKOV, 1992). Utilization of chelating compounds helps to balance availability of micronutrients for the plant root system. The potential answer for this can be the application of different substances of organic nature that due to their structure are capable of retaining macro and microelements, making them available for the rhizosphere. The influence of the top dressing fertilizer Lactofol"O"® on the development of soil and epiphytic microflora was investigated. Lactofol"O"® was used alone, or in combination with NPK. The total number of microorganisms was determined by dilution agar plate. The suspension fertilizer Lactofol"O"® slightly inhibits the development of bacteria and fungi in French bean cv. Helda, and stimulates the development of actinomycetes. This top – dressing fertilizer exerts a more favorable effect on

the rhizosphere microorganisms in French bean cv. Trakiiski as well as the epiphytic microflora (SAPUNDJIEVA *et al.,* 1997).

#### 2.2.3.2 Effects of lactates

As primarily chelating substance artificially introduces into soilless culture Lactofol"O"® fills the following roles:

***1. Increase the availability of nutrients.*** Chelating attributes of LACTOFOL "O" is capable of creating bonds with the relatively insoluble iron, under high pH-values in substrate and make it available to plants.

***2. Prevent mineral nutrients from forming insoluble precipitates.*** The chelating agents of the metal ions will protect the chelated ions from undesired chemical reactions and hence increase the availability of these ions to plants. An example of such reaction is the behavior of iron in substrates with high pH. In soil with high pH, iron will react with hydroxyl group (OH-) to form insoluble ferric hydroxide ($Fe(OH)_3$), according to reaction shown below, which is not available to plants (HAJRA and DEBNATH 1985). Chelation will prevent this reaction from happening and hence render iron available to plants.

$$\xrightarrow{\text{pH>7}}$$

$$Fe^{+3} + 3\ OH^- \dashrightarrow Fe(OH)_3 \quad (2)$$

*Soluble* *Insoluble*

***3. Reduce toxicity of some metal ions to plants.*** Chelation in the substrate may reduce the concentration of some metal ions to a non-toxic level. This process is usually accomplished by humic acid and high-molecular-weight components of organic matter.

***4. Prevent nutrients from leaching.*** Metal ions forming chelates are more stable than the free ions. Chelation process reduces the loss of nutrients through leaching (SCHNITZER and SKINNER, 1967).

***5. Metabolism enhancement.*** Suspension of nutrient elements as well as Lactate itself is proved to be an effective metabolism enhancer in agricultural plants as well as microorganisms. Leaf fertilizer Lactofol"O"® inhibits the development of bacteria and fungi in French bean *cv.* Helda, and stimulates the development of actinomycetes. Lactofol"O"® contributes to formation of better microbiological conditions in the rhizosphere microorganisms population in French bean cv. Trakiiski as well as the epiphytic microflora (SAPUNDJIEVA *et al.,* 1997).

### 2.2.4 Description of complex or combined biostimulators

Biostimulators in agricultural production have long been regarded as a mean for dealing with suboptimal growth conditions (BÖHME *et al.,* 2007). Applying biostimulating agents, we increase intensity of metabolic processes that had been hampered by stress factor(s). In this sense biostimulators and plant strengtheners can be considered as part of one classification system (Figure 2.6). Application of plant strengthening substances in horticultural production is called upon to improve plants' growth and thus contribute to sustainability of production. Biostimulator in this case is a substance, biological agent that harnesses plant's defense mechanisms.

Definition of plant strengthener states that it can be a chemical compound or microorganism that is capable to protect plants against malicious organisms through triggering the defense mechanisms of the plant by: - stimulating resistance/defense mechanisms in the plant, or – the competition of the plant strengthener with harmful organisms for space and food-substances in the fyllosphere or rhizosphere (EUROPEAN COMMISSION DIRECTIVE 91/414/EEC 2001). Application of plant strengthening substances in horticultural production is dedicated to improve plants' growth and thus contribute to sustainability of agricultural production. Biostimulator in this case is a substance, a biological agent that supports plant's defense mechanisms.

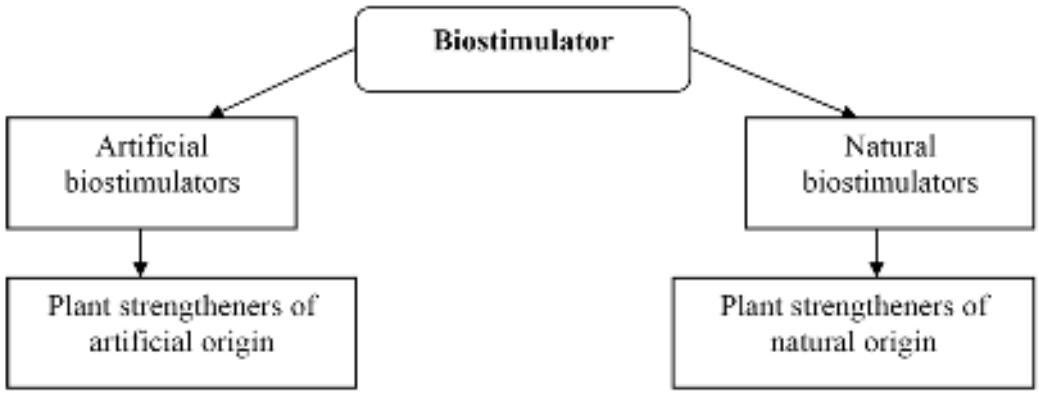


**Figure 2.6** Relation between biostimulators and plant strengtheners

Plant strengtheners are defined in the German Plant Protection Act Article 2 no. 10. According to it plant strengthening agents are at the same time plant resistance improvers and possess the following characteristics:

- Plant resistance improvers are to enhance the resistance of plants to harmful organisms;
- Intended to protect plants against non-parasitic impairments.

According to these definitions plant strengtheners are used mainly for control of microbial community in the rhizosphere or phyllosphere. Problems of biostimulators application in general can be summarized as follows:

- Sustainability of performance (*B.subtilis* requires constant level of moisture in the substrate; otherwise it does not evolve its positive function).
- Specific concentration is needed for particular cultivar and particular purpose of application (breaking seed dormancy requires higher concentrations of biostimulators).
- Every biostimulator has its specific spectrum of action.

Following from these limitations and problems of both biostimulators and plant strengthening agents a conclusion can be made that for achieving of most positive results in stress reduction and overall improvement of plant's ontogenesis the different biostimulators and plant strengtheners can be applied in combinations. Combinations of these agents must serve as supplement to each other creating cohesive effect on plant.

Humates are substances that confer on plants different positive effects but in many cases for every positive result achieved through humate application another one with negative sign can be found. The reason for this is confined in activity spectrum of humates. Influencing growth of root system and uptake of nutrient elements, humates may also be a substrate for microbiological activity – both malign and favorable for plants. To reduce negative effects, introduction of beneficial bacteria might be considered. Application of *B. subtilis* FZB 24® leads to the following processes in the root system of the plants: antibiosis with regard to other microorganisms, competition among microorganisms for plant's exudates, induced resistance through production of polysaccharides in rhizosphere, growth promotion of plants, increased growth favors development of ample yield, disease escape, and improved overall plant strength. Lactofol"O"® designed primarily as foliar fertilizer but can also be applied in the root area. As a carrier of micronutrients it is indispensible for seedlings. It can be also used to sustain plants on different stages of their development. Application of this substance during the blossoming phase of plant's development contributes to higher fruit formation.

Considering all positive sides of each substance and their shortcomings, an inference can be made that application of these substances in the mixture may have much more positive effect on plants rather than their singular application.

# 3 The problem statement

## 3.1 Problem description

In protected cultivation especially in hydroponics, suboptimal growing factors can arise from different factors of horticultural production. In many cases suboptimal or stress factors manifest themselves through a variety of physiological reactions that can be visually observed. These reactions often lead to phenotypic changes like chlorosis, discoloration, suppressed growth and development.

At the same time abiotic stress, before expressing itself in a visual form, triggers a variety of physiological reactions (ÖQUIST and STRAND, 1988). That is why in many cases it is impossible to determine exact onset of particular stress before it occurs. Plant strengtheners possibly can support plant growth also in hydroponics to counteract main problems in pH- EC and nutrient balance. They are capable to reduce different stresses in hydroponics as previous experiments indicated. Lactates, salts of lactic acid, can be used to chelate nutrients, especially micronutrients. K-Humate – improves growth of roots and facilitates uptake of nutrient elements from the substrate. *Bacillus subtilis* FZB 24® – suppresses malicious microflora in the root area of the plant. Stress reducing effects of lactates could be found especially in nutrient solutions with too low or too high pH values and also in stress situations because of extreme temperature (BOEHME *et al.,* 2000). It is significant to develop a mixture of biostimulating substances that can be applied at the start of the vegetation and evolve its effects in the process of plant's growth.

Existing knowledge about interaction between plant strengtheners and immediate environment are not sufficient enough. Scientific data especially on application of lactates in horticulture are sketchy at best and do not give answer to many significant questions of horticultural practice.

The major elements of the current study are – horticultural plants, three biostimulating substances K-Humate, Lactofol "O", *Bacillus subtilis* FZB 24® and growing substrates. The process of horticultural plants cultivation brings about different mechanisms of interaction of each element involved (Figure 3.1).

It should be find out if such combination of these substances supports their beneficial effects or if they interfere with each other. The biostimulators can be applied in the root zone or on the leaves. It should be investigated which treatment is the most effective one. To probe the theory of the biostimulating effect of a mixture of all three substances, it is necessary to conduct research on most commonly used horticultural substrates like perlite, rockwool, peat, coir and somewhat less common – sheep wool.

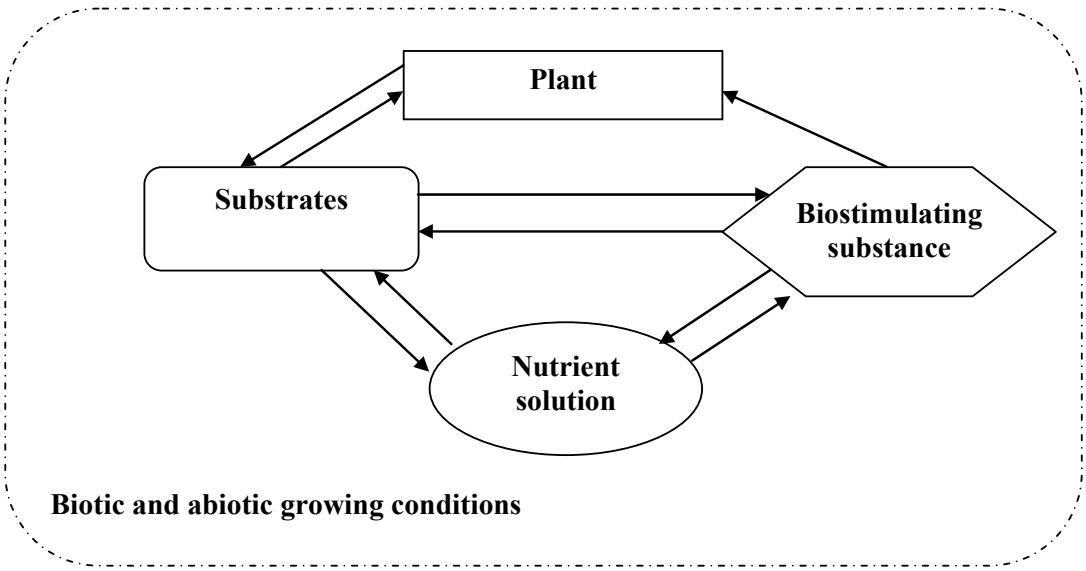


**Figure 3.1** Interactions of different elements in the growing system

To probe the theory of the biostimulating effect of a mixture of all three substances, it is necessary to conduct research on most commonly used horticultural substrates like perlite, rockwool, peat, coir and somewhat less common – sheep wool. Using some of these substrates in experiments with plant strengthening compounds can bring different results primarily because of different nature of these substrates.

## 3.2 Objective of the research

The objective of this study is the investigation of the influence of humates, lactates and *B.subtilis* FZB24 on growth and yield of *Cucumis sativus L.* This scientific investigation researches on application of humates, lactates and *B.subtilis* FZB24 in horticultural production. In addition, this work investigates physiological interactions between plant, substrate and application of biostimulating mixture of K-Humate, Lactofol"O" and B.subtilis FZB24.

## 3.3 Hypothesis of the study

Based on scientific evidences and experiences with substrates and biostimulating substances, there is a possibility to set out the following hypothesis of this study:

**Hypothesis 1:** Application of lactates, humates and *Bacillus subtilis* FZB 24® as a mixture is much more preferable in comparison to separate application of these substances. Their combined

application in critical stages of plant's development provides better growing conditions and increases plant's vitality.

**Hypothesis 2:** Interaction of humates, lactates, microorganisms with horticultural substrates are coherent and not competitive. Substances applied together implement their function and hence have a broader efficacy spectrum.

**Hypothesis 3:** Application of biostimulating mixtures can have different effects as a result of different properties of substrates as well as different ways of application of biostimulating mixture.

**Hypothesis 4:** Biostimulating mixture that consists of K-Humate, Lactofol"O"®, *Bacillus subtilis* FZB 24® is assumed to have stress relieving effect, on plants that have undergone periods of suboptimal growth conditions.

**Delimitations of the study.** Problems addressed in this research have to do with plants reactions on biostimulating substances and plant strengthener. Application of lactates, humates and *B.subtilis* triggers a variety of physiological effects in plants. The whole complexity of these interactions cannot be described, nor was it a purpose of this study. Instead, there is a focus on such physiological reaction like "electron efficiency of photosystem II", that describes physiological status (under stress/no stress) of the plants. Humates, lactates, *B.subtilis* that are used in this research are commercially available substances with history of scientific trials. Concentrations of substances used in this research are based on recommendations of particular producer of the substance. Current study goes into questions closely related to the field of microbiology but it is not a microbiological study and microbiological research has never been a purpose of it. Experiments are conducted in greenhouses and climate chambers which have limited capacity and thus limited number of plants and thus number of variants and repetitions. To test effectiveness of biostimulating mixtures, research is dealing with evaluation of abiotic factors in terms for stress resistance test of the plants. There are a limited number of suboptimal growing factors that were tested.

### 3.4 General research pathway

The research starts with testing the role of iron-humates of different origin on cucumber plants with and without iron deficiency in nutrient solution. This experiment answers the question which of iron-humates brings most positive effects on cucumber plants in terms of productivity. Later experiments with iron-humate and potassium-humate address the question of better application of these substances. The way of application (leaf application and root application) is

a crucial question for later experiments. Figure 3.2 describes the general pathway and stages of the research.

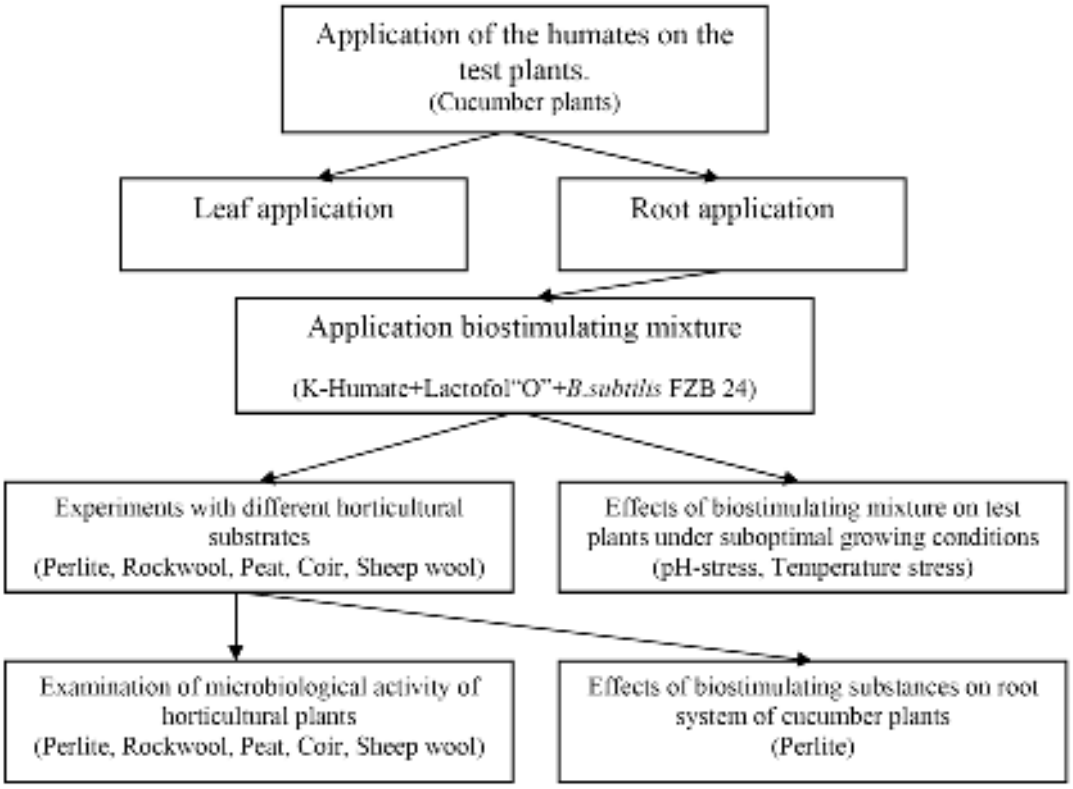


**Figure 3.2** The general scheme of the research pathway

The mixture of *B.subtilis* FZB 24, K-Humate and Lactofol "O" was tested on different horticultural substrates. Substrates were used during four vegetations. Application of biostimulating mixture on plants under suboptimal growing conditions in the climate chamber proved its stress-relieving capabilities. After long-term utilization substrates were tested on microbiological activity by employing substrate-induced respiration SIR-method.

# 4 Materials and methods

## 4.1 General plan of the research complexes

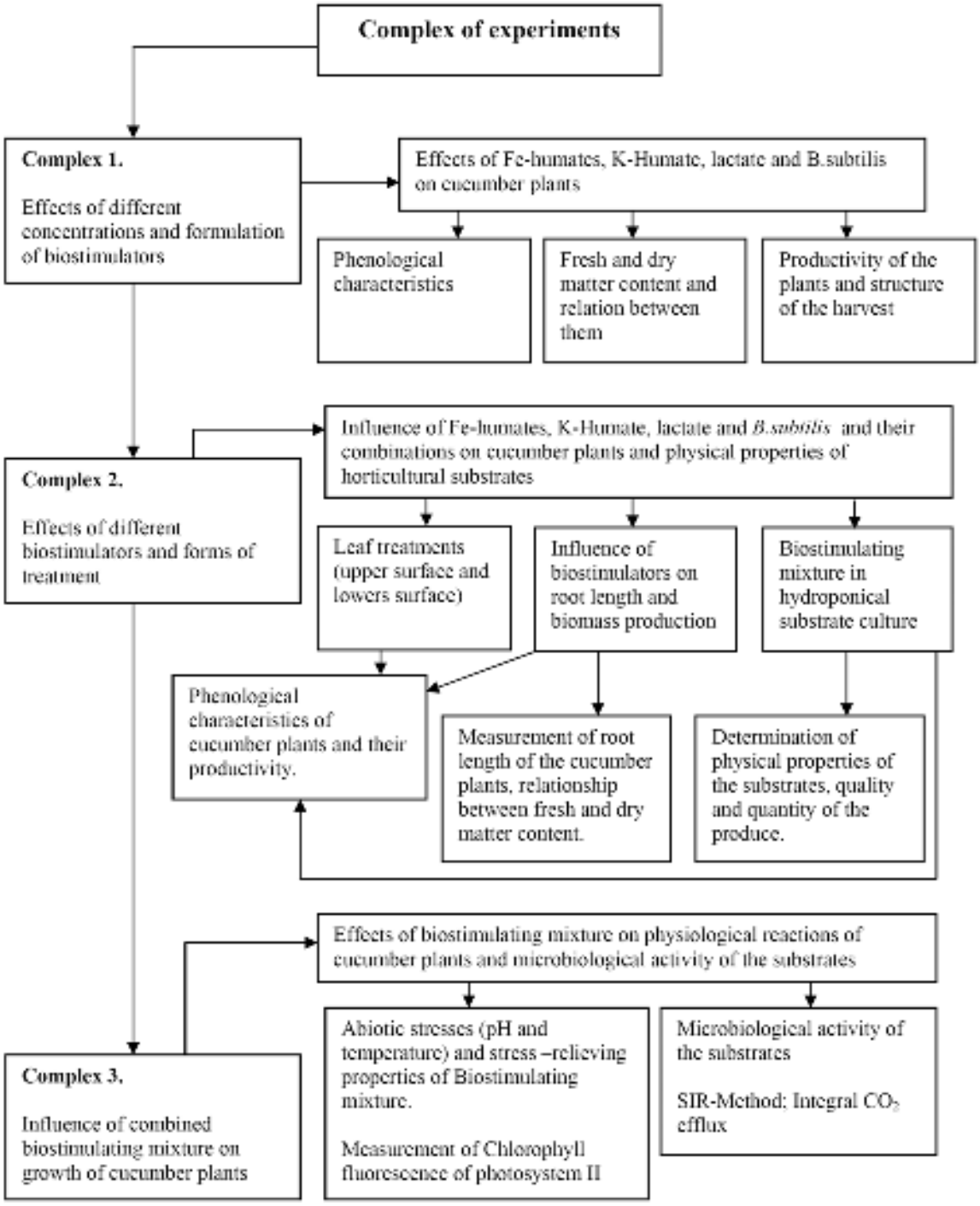


**Figure 4.1** The general description of the experiments within separate research complexes

### 4.1.1 Plant material and major operations

In the course of this research through the years 2002-2006 plants of *Cucumis sativus* L. cv. Jessica (F1) and cv. Indira (F1) were used for the experiments. Major operations within complexes are described in table 4.1.

**Table 4.1** Major operation within research plans

| Research Complex | Experiment | Key research point | Cultivar | Sowing | Picking | Planting | First harvest | Last harvest | End |
|---|---|---|---|---|---|---|---|---|---|
| 1 Complex | 5.1.1 | Fe-Humate applied with standard nutrient solution and solution with iron-deficiency | Jessica (F1) | 07.10.02 | 12.10.02 | 28.10.02 | 02.01.03 | 15.03.03 | 16.03.03 |
| | 5.1.2 | Humate Lactofol *B.subtilis* | Jessica (F1) | 15.03.03 | 25.03.03 | 25.04.03 | 05.05.03 | 30.08.03 | 03.09.03 |
| 2 Complex | 5.2.1. | Humates leaf treatment | Jessica (F1) | 12.01.03 | 25.03.03 | 28.01.03 | 15.03.03 | 26.07.03 | 26.07.03 |
| | 5.2.1. | Humate Lactate *B.subtilis* Leaf/root treatments | Indira (F1) | 20.03.03 | 27.03.03 | 15.04.03 | 20.05.03 | 25.07.03 | 26.07.03 |
| 3 Complex | 5.3.1. | Biostimulating mixture K-Humate Lactate *B.subtilis* *First rotation* | Indira (F1) | 23.09.03 | 12.10.03 | 18.11.04 | 26.02.04 | 28.04.04 | 30.04.04 |
| | | Biostimulating mixture K-Humate Lactate *B.subtilis* *Second rotation* | Indira (F1) | 24.05.04 | 30.05.04 | 10.06.05 | 12.07.04 | 01.11.04 | 05.04.04 |
| | | Biostimulating mixture K-Humate Lactate *B.subtilis* *Third rotation* | Indira (F1) | 25.11.04 | 14.12.04 | 12.01.05 | 15.02.05 | 14.04.05 | 15.04.05 |
| | | Biostimulating mixture K-Humate Lactate *B.subtilis* *Forth rotation* | Indira (F1) | 20.05.05 | 29.05.05 | 9.06.05 | 11.07.05 | 15.11.05 | 25.11.05 |
| | 5.3.2. | Biostimulating mixture Root length and Biomass | Indira (F1) | 16.01.06 | 22.01.06 | 25.02.06 | 25.03.06 | 15.06.06 | 17.06.06 |
| | 5.3.3. | Biostim mixture and pH (alk) stress | Indira (F1) | 05.08.04 | 15.08.04 | 20.09.04 | - | - | 15.12.04 |
| | | Biostim mixture and pH (acid) stress | Indira (F1) | 15.12.04 | 25.12.04 | 15.01.05 | - | - | 20.04.05 |
| | | Biostim mixture and Temp. stress | Indira (F1) | 09.02.05 | 13.02.05 | 02.03.05 | - | - | 15.05.05 |
| | 5.3.4 | Microbiological activity of substrates | Indira (F1) | 15.05.05 | | | | | 10.10.05 |

The hybrids were delivered by Rijk Zwaan seed company. The hybrid **cv. Jessica** was used in the first two experiments. Due to its susceptibility to mildew, this cultivar was changed for cv. Indira (F1).

**Cultivar Indira (F1)** is characterized by a high yield potential and capability to deliver sustainable, uniformed fruits even from lateral shoots of the plant's stem. It can be used for obtaining yields of cucumber fruits under circumstances of suboptimal light conditions, which can be the case during winter times.

**Young plant production.** All plants for experiments were started from the seeds in perlite substrate in the plastic tray of 30x75 cm and at this period they were watered with tap water at temperature of 25-27°C. The cucumber plants planned for rockwool and sheep wool as the substrates were started directly in the rockwool cubes. The plantlets of cucumber cultivars were transferred to the substrates at the development phase of 3-4 leaves.

**Plant management**

Plants planed for cultivation in substrates like sheep wool, rockwool, peat, and coir were transferred to rockwool cubes of 10x10x10 cm size. During this period plants were watered with nutrient solution with EC value of 1.5-1.7 mS * $cm^{-1}$. At the age of 3-4 weeks (4-5 leaves) plants were transferred into permanent cultivation pots (containers, substrate slabs, etc). After transfer plants were supplied with nutrient solution of the same composition but higher EC value – 2.0-2.1 mS * $cm^{-1}$.

**Pruning**. Beginning from 5th week period plants start to develop flowers. To improve productivity of the plants, all first flowers and tentacles were removed up to 50 cm during the summer and 70 cm during the winter period. First harvest was collected before fruits ripened into marketable value. During vegetation plants were checked for diseases and pest on daily basis. Harvesting started depending on ripening stage of cucumber fruits. Distorted fruits and fruits with signs of diseases were removed. The test plants were cultivated in substrate culture using different kinds of horticultural substrates that are described in paragraph 4.5. Test plants were grown on the vegetation tables using Mitscherlich-Pots 8 l.

### 4.1.2 Greenhouse

All experiments in the current research were conducted under controlled conditions of the greenhouse. Separated compartments of the greenhouse allowed regulating air temperature, substrate temperature, relative air humidity, light conditions. These climatic parameters were read daily at 8 a.m. and 2 p.m. Temperature ranged between 22°C minimum and 29°C maximum

and air humidity was between 70 and 80%. Average temperature during the day was about 28.3°C and during the night 23.1°C. These values represent the average of three separate data readings. The most deteriorating factor was air temperature during the night. Regarding that optimal temperature conditions for cucumber plants cannot be lower then 24-25°C, it can be stated that test plants in some experiment were subjected to suboptimal growing conditions. Additional thermometers in the substrates made it possible to monitor maximum and minimum temperature during 24 hours.

The water-heating pipelines were arranged around the perimeter of the chamber of the greenhouse with additional pipes being arranged alongside the cultivation tables and cultivation beds. Air conditioning system was automatic and kept air temperature and relative air humidity within preset parameters of their maximum and minimum. Additional light conditions were created during the winter periods using lamps of type PL-90 E with power of 120 W * $m^{-2}$. The duration of additional irradiation by Na-lamps was set at 7:00-19:00 hour to guarantee light conditions equal to 10 Klux.

### 4.1.3 Climate chamber

Climate chamber HPS 1500 from "Heraeus" company was used for the experiments with abiotic stress factors. The inner volume of the chamber measures 1500 liters. During experiments in the climate chamber following characteristics were maintained:

PAR = 500

Relative air humidity 80%

Temperature day/night = 25/26°C.

## 4.2 Biostimulating substances and plant strengtheners

The experiments aimed at testing plant's response on treatments with different humates, Lactofol "O", *Bacillus subtilis FZB* 24. For this purpose, different combinations of these substances were tested on cucumber plants.

**Plant strengthener**

***Bacillus subtilis FZB* 24®** has been developed by FZB Biotechnik GmbH and is marketed in Germany by Bayer as a plant-strengthening agent. It was used for root and leaf applications of cucumber plants on different horticultural substrates.

**Humates**

**K-Humate.** Commercially available product of Humintech GmbH, Germany. K-Humates is water soluble with neutral pH reaction. This humate is extracted from brown coal and is commercially available. Concentration of the humates in the aggregate material was 85% Concentrations for solution preparation have been worked out in previous researches and taken for current experiments 0.001, 0.005 – 0.2 %. Concentration of K-Humate in the long-term experiments was 0.01%. This concentration is resulted from the recommendation of the manufacturer of the substance.

**Fe-Humates.** Two types of HUMIRON were compared; one type contains humic acid extracted from a Russian brown coal (HUMIRON® type R) and the other humic acid from a German brown coal (HUMIRON® type G). Both humate types contain 7% of iron in its composition.

**Lactate**

**LACTOFOL "O" ® -** Salt of lactic acid – suspension of nutrient elements used for foliar fertilization. Composition of Lactofol is presented in table 4.2.

**Table 4.2** Composition of LACTOFOL "O" ®

| Components | Unit | LACTOFOL | Components | Unit | LACTOFOL |
|---|---|---|---|---|---|
| Lactic acid | % | 10 | Magnesium | % | 0.1 |
| Riboflavin | mg $l^{-1}$ | 0.5 | Iron | % | 0.4 |
| Ascorbic acid | mg $l^{-1}$ | 3 | Boron | mg $l^{-1}$ | 300 |
| Thiamine | mg $l^{-1}$ | 0.1 | Copper | mg $l^{-1}$ | 200 |
| Nitrogen | % | 30 | Manganese | mg $l^{-1}$ | 250 |
| Phosphorus | % | 7.5 | Zinc | mg $l^{-1}$ | 125 |
| Potassium | % | 15 | Molybdenum | mg $l^{-1}$ | 18 |
| Calcium | % | 0.5 | Cobalt | mg $l^{-1}$ | 6 |

**Preparation of biostimulating mixture**

The mixture was composed of *B.subtilis* FZB 24, K-Humate and Lactofol"O". Preparation of *B.subtilis* FZB 24 solution was implemented according to specification for this product. A weighted quantity of *B.subtilis* FZB 24 (2 gram) was added to the 1l of tap water of with temperature of 45-50°C. The solution was constantly stirred till the temperature reached 25°C. This manipulation resulted in 0.2% concentration of *B.subtilis*.

Solutions of humates were prepared by weighting a specific quantity of the salt with subsequent dilution in measured volume of deionized water. The solution was stirred till there were no

visible particles of the humic acids in the suspension. All humates used in this study are water-soluble.

The targeted concentration of Lactofol "O" 0.1% was prepared by measuring a volume of Lactofol "O" and diluting it in 1l of deionized water (v/v). The obtained solutions of humate, lactate and *B.subtilis* were mixed in equal parts (1:1:1). The biostimulating mixture in this formulation had unsuitable pH values of 8.7-9.5. It was readjusted by adding several drops of 75% $H_3PO_4$ into the solution.

Application of biostimulating substances was performed in two manners. The leaf application was accomplished through spraying of the solution onto the phyllosphere of the test plants. The solution for the leaf application with concentration 0.05% (w/v) HUMIRON-G® and K-humate was used. The root application was achieved by watering of the solution into the rhizospheric area of the test plant.

### 4.3 Horticultural substrates

Several horticultural substrates were used for experiments in this study (Figure 4.2).

**Perlite.** Average dry density of 120 kg * $m^{-3}$ was used. The grain size was between 0.06mm and 1.5mm, with 45% of all grains having a diameter of 1 mm. Pore volume was 90.2 ± 0.82% v/v, the water holding capacity was 31.6 ± 1.51% v/v, and the air capacity was 58.6 ± 1.51%.

**Rockwool**. The rockwool slabs measured 7.5 cm x 15.0 cm x 90 cm or a volume of slightly more than 10 liters. Pore volume at the beginning of experiments was 90.7 ± 0.83% v/v, the water holding capacity was 41.6% ± 10.25 v/v, and the air capacity was 49.2 ± 9.98%. Rockwool slab accommodates two plants.

**Coir**. Coir is a coconut dust residue that constitutes mesocarp of the coconut fruit (*Cocos nucifera*). Coir dust is widely used as a substrate culture. The total volume of medium supplied is 12 liters and have a size of 15.0 cm x 90 cm. Pore volume was 83.9 ± 3.42% v/v, the water holding capacity was 52.8 ± 2.27% v/v, and the air capacity was 30.6 ± 2.37%. Every sack of coir has place for two plants.

**Peat**. The aggregate volume of medium is about 12 liters. Size of peat slab is 8 cm x 15.0 cm x 95 cm. Pore volume was 86.0 ± 2.82% v/v, the water holding capacity was 68.0 ± 3.55% v/v, and the air capacity was 18.0 ± 1.82%.


**Figure 4.2** Horticultural substrates used in the study

**Sheep wool**. This substrate is a byproduct of sheep husbandry. It is used in the experiments as a novel horticultural substrate. The substrate was provided as pressed rolled up sheep wool**.** Pore volume was 96.8 ± 1.30% v/v, the water holding capacity was 22.8 ± 1.72% v/v, and the air capacity was 69.4 ± 1.51%. Coconut fiber is used to improve drainage in the substrate.

## 4.4 Nutrient solution

The standard nutrient solution (BOEHME, 1993) was used with complete macro- and micronutrients (170 ppm N, 50 ppm P, 260 ppm K, 150 ppm Ca, 60 ppm Mg, 3 ppm Fe, 90 ppm $HCO_3$, 80 ppm S). For seedling and plantlets the same composition of the nutrient solution was used although with lower EC value of 1.6-1.8.

**Irrigation.** Nutrient solution was delivered to plants by trickle irrigation. The quantity of the nutrient solution was automatically adjusted to the intensity of solar radiation. In the summer when consumption of the nutrient solution is at its height, additional water supply was arranged. The containers were irrigated with a trickle irrigation system. 'Netafim' drippers with a capacity of 2 l $h^{-1}$ were used. The plants were irrigated 2 to 4 times a day and 150 ml per irrigation cycle was applied in periods of 10-12 min.

## 4.5 Determination of growth and yield parameters

**Plant evaluation.** Plant growth: plant's height – weekly; number of leaves – weekly.

**Leaf area estimation**. One of the most important parameters of the plant's development is formation of the assimilation apparatus. Monitoring of the leaf area development during vegetation was conducted by measurement of the leaf length (L) and leaf width (W) (Figure 4.3). Only true leaves were selected for data reading. The first untrue leaves and leaves during initial stage of their development on the shoot tips were discarded from the measurement. The shape of the leaf was approximated to the form of the rectangle. The measurement of both length and width of the leaf was taken between farthermost points that lie on the sides of the rectangle.

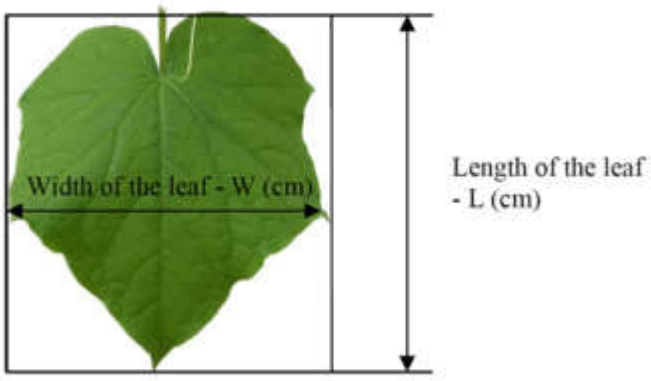


**Figure 4.3** Principle of the leaf area estimation

The formulae for calculation is designed for three cases – first is when the length of the leaf is greater then its width (1), the second – when the width of the leaf is greater then its length (2) and the third one, when width and length of the leaf are equal (3) (BOEHME, 1994).

$$\text{Leaf area } (cm^2) = L \times W - (0.06 \times L \times W) \quad (1);$$

$$\text{Leaf area } (cm^2) = L \times W - (0.04 \times L \times W) \quad (2);$$

$$\text{Leaf area } (cm^2) = L \times W - (0.19 \times L \times W) \quad (3);$$

The leaf area data were obtained for every leaf and then expressed as arithmetic sum for every tested plant. Number of leaves was counted weekly on the same day with measurement of the shoot length. Determination of root length of the plants as well as fresh and dry matter content was conducted after each experiment.

**Root length measurement.** Roots of the plants after vegetation were removed from the substrates. The roots are washed from the substrate quantitatively and transferred on the surface

of the root length scanner "Comair". The reading of the root length data is taken after the scanner stops estimation.

**Harvesting and cucumber fruit evaluation.** The harvesting was conducted according with fruit condition and ripening. The first harvest was conducted before fruits achieved marketable parameters (Table 4.3). Fruits were plucked and sorted according their variant and replication then fruits were weighed measured in their length and diameter. The cucumber fruits were evaluated according to their appearance into marketable and non marketable.

**Table 4.3** Criterion for cucumber fruit evaluation

| Marketability | Class | Criterion |
|---|---|---|
| Marketable fruits | Extra | Good developed. Good shaped. Practically direct without curving. Maximal curving 10 mm for every 10 cm of fruit length. Impeccable color. Absence of any misshapes.<br>Fruits that weigh below 500 g have to be at least 25 cm long, fruits that weigh more then 500 g should be at least 30 cm long. |
| | A | Sufficiently developed fruits, good shaped, practically without curving. Slight misshape is acceptable. Aberration of color is acceptable especially at the place of their contact with the substrate. Maximal curving 10 mm for every 10 cm of fruit length. Fruits that weigh below 500 g have to be at least 25 cm long, fruits that weigh more then 500 g should be at least 30 cm long. |
| | B | Color aberration maximum 1/3 of the surface. Slight damage of the surface. Moderately curved cucumbers 20 mm curving for every 10 cm of the fruit length. |
| Non-marketable | C | Misshaped fruits with substantial discoloration. Parameters of "Extra", "A" and "B" classes are not confirmed. |

**Dry Matter Content determination.** The dry matter content was determined for leaves and stems of *S. rebaudiana* in *in vitro* meristem and sprout culture respectively. The leaves and stems were weighed to the nearest 0.001g. The record of moist sample weight was taken (m1). The aluminum pan tare for every sample was weighed and recorded (m*t*). The samples were transferred into the preheated drying oven at 105°C for 24 hours. The dried samples were removed to desiccators for 2 hours. The dried plant material was removed from the desiccators and weighed to the nearest 0.001 g. The record of dried sample with the pan tare was taken (m2*t*). The dry matter content of the samples was calculated according to formula:

$$\text{Sample dry matter (\%)} = \frac{[1 - \text{m1} - (\text{m2}t - \text{m}t)]}{\text{m2}t} \text{ x } 100; (4)$$

The result was recorded to the nearest 0.1%.

## 4.6 Chemical methods

**Determination of nitrate in plant material.**

*Measurement* of nitrate content was conducted using a method of direct potentiometry. The nitrate selective electrode was used in combination with the Microprocessor pH/ION Meter pMX. The calibration was performed using two point calibration methods with nitrate standard solutions of 50 and 500 mg $l^{-1}$.

*Sample preparation*: The nitrate estimation in the cucumber fruits were conducted by selecting of 3 cucumber fruits from each replication of the variant. The sampled fruits were grinded, and the fruit mass of these three cucumbers was mixed together to obtain a mixed sample. The amount of 10 gram of the mixed sample was placed into 50 ml marked beaker and filled with deionized water to the 50 ml volume mark. The results of the nitrate content were read from the pH/ION Meter pMX in 1 minute after the nitrate selective electrode was delved into the sample solution in the beaker. The samples in which the nitrate content was measured had been pre-conditioned by adding 1 ml TISAB/$NO_3$ per 50 ml directly into the sample solution. Determination of nitrate content in the leaves and stems was conducted as follows: 100 gram of the fresh matter were taken from every replication and placed into 1 liter volumetric flask. The fresh matter of particular repetition was used to prepare a mixed sample by blending the fresh matter to the consistency of a thin slur. The deionized water EN ISO 3696 was taken to bring total volume in cylinder to 1 liter.

*Measurement*. Results are read after one minute of stirring by magnet rod. Mineral content of the cucumber plants was determined in central lab of agricultural department of Humboldt-University. Determination of the potassium, magnesium and calcium was conducted using the method of atomic-absorption spectrophotometry.

**pH and EC measurements**

**Nutrient solution**: Standard nutrient solution. Plants were watered with 200 ml of nutrient solution daily. For monitoring pH and EC values as well as nitrate content in nutrient solution a mix sample was prepared by sampling 100 ml of nutrient solution from every plant in the variants and mixing it together. After that 100 ml of sample was taken for filtration with subsequent determination of pH, EC and N-$NO_3^-$. The reading of pH and EC parameters of nutrient solution was conducted using portable pH-meter “Combo pH&EC” of HANNA GmbH. Element Content (N-$NO_3^-$ , $K^{2+}$, $Ca^{2+}$, $Mg^{2+}$) – nutrient solution (harvesting period) was determined using ion selective electrodes and Microprocessor pH/ION Meter pMX 3000.

pH-values were sampled daily by taking samples of nutrient solution (80 ml). pH values were 6.5-6.7. After filtration of sample EC Value was determined 1.9-2.2 mS * cm. The salt concentration (EC) in the nutrient solution was between 2.0 and 2.4 mS * cm-², the pH value ranged from 5.8 to 6.5.

**Analyses of nutrient content in substrates**

Principle of ion-selective electrode calibration presented in table 4.4. Contents of nitrate and potassium were evaluated in the different substrates. Elemental analysis in perlite and rockwool was confined to the determination of the nutrients in their respective drainage waters.

**Table 4.4** Analysis in the substrates

| Nutrient | Calibration | Two point calibration |
|---|---|---|
| *nitrate* | 10 g $l^{-1}$ $NaNO_3$ | 50 mg $kg^{-1}$ and 100 mg $kg^{-1}$ |
| *potassium* | 10 g $l^{-1}$ potassium chloride | 20 mg $kg^{-1}$ and 200 mg $kg^{-1}$ |

*Sample preparation*: 50 g of sieved substrates sample is placed into 150 ml vessel. 100 ml of the deionized water was added to the substrate sample. 2 ml of the conditioning solution was measured into the watery mixture of the substrate. Sheep wool sample of substrates was cut in pieces 20-30 mm long. Rockwool samples were not analyzed; instead discarded nutrient solution was used for analysis. The samples were put on a shaker for 30 minutes with subsequent filtration afterwards. The filtrate was used for determination of nitrate and potassium content in the substrate. The calibration of the ion-selective electrodes is showed in the table 4.5.

**Table 4.5** Analyses in nutrient solution

| Nutrient | Calibration | Two point calibration |
|---|---|---|
| *nitrate* | 10 g $l^{-1}$ $NaNO_3$ | 50 mg $kg^{-1}$ and 500 mg $kg^{-1}$ |
| *potassium* | 10 g $l^{-1}$ potassium chloride | 20 mg $l^{-1}$ and 200 mg $l^{-1}$ |
| *calcium* | | using 20 mg $l^{-1}$ and 200 mg $l^{-1}$ |

*Sample preparation*: 100 ml of nutrient solution is placed into 150 ml vessel add 2 ml of conditioning solution TISAB/$NO_3$. Reading is taken in one minute after stirring with magnetic rod.

## 4.7 Methods to estimate the physical properties of growing media

Samples of substrate material are taken from every variant to evaluate its physical properties. Sampling is conducted after the end of every rotation using VDLUFA methodology. 200 g of substrate material (case of perlite, coir and peat) was sampled from every repetition of the variant. For analysis of rockwool, entire slab of the first repetition is removed. Sheep wool was taken for analysis by removing all substrates material. Subsequently rockwool slab and sheep wool are substituted in the experiment by the new (fresh) substrate material. After the next rotation, the sample of rockwool is taken from the slabs that previously were not substituted. The structural properties of horticultural substrates were analyzed by air-pycnometer (KUNZE, 1942). Being modified by Geyer, Großkopf and Stracke (1972) the new version of this apparatus (Figure 4.5) used for measurement of such parameters as air capacity, water holding capacity.

**Figure 4.5** Modified air-pycnometer

Determination of structural characteristics and physical properties of the substrates with air-pycnometer (BÖHME and VORWERK, 2003):

1. A horticultural substrate was filled into a Cylinder with volume of 500 $cm^3$. (CV)
2. Cylinder filled with a substrate was weighed and by calculating differences of masses between empty and filled cylinder the mass of the substrate was obtained. ($W_1$)
3. The cylinder was put into the pycnometer, by activation of the lever of the pycnometer the situation was achieved when quicksilver substitutes the air in the cylinder. The value was recorded (PW).
4. The cylinder was covered with a thin net and put into a bath flooded with water (for 24 hours). The cylinders were taken out and after water was drained the weighed again ($W_2$)
5. The samples were dried in the drying cabinet and the mass of the dried samples was read ($W_3$).
6. Calculation of the water holding capacity $WC=W_2-W_3$
7. Calculation of the water content $WG= W_1-W_3$ in g 500 $cm^{-3}$
8. Calculation of the pore volume PV=CV – PW + WG

9. Calculation of the air capacity AC=PV-WC
10. Determination of hard particles FB=CV-PV
11. Determination of the air content LG=CV-PW
12. Determination of bulk density D=W3/CV

For the purpose of the physical properties description of the horticultural substrates only parameters of AC – air capacity, WC – water capacity, PV – pore volume are used.

## 4.8 Experiments in the greenhouse

### 4.8.1 Conditions of vegetation experiments

**Conditions for experiment: "Effects of iron-humates on cucumber plants in substrate culture". (Subsection 5.1.1)**

**Plant material and growing conditions.** The aim of the experiments was to investigate the effect of different applications of the soluble Fe-humate (HUMIRON®). The application was conducted by applying the humate solution into the rhizosphere. The rhizospheric application was compared to foliar application of the humate. The different treatments had effects on the growth and yield of cucumbers (*Cucumis sativus* cv. Jessica (F1)). Two types of the humates were used in the experiment. HUMIRON® type R (extracted from Russian coal) and G (extracted from German coal) were compared. Three different concentrations (0.001%, 0.1%, and 0.2%) of HUMIRON® were used. Cucumbers were grown in a substrate culture used containers with 8 l perlite. Nutrient solution with and without iron was applied with trickle irrigation. The scheme of the experiment is presented in table 4.6.

**Table 4.6** The scheme of the experiment

| **Biostimulators** | | **Nutrient solution** | |
|---|---|---|---|
| **Compound** | **Concentration** | **Standard** | **Without iron** |
| | 0 (control) | X | X |
| HUMIRON Fe 8% (R) | 0.001% | X | X |
| HUMIRON Fe 8% (R) | 0.1% | X | X |
| HUMIRON Fe 8% (R) | 0.2% | X | X |
| HUMIRON Fe 8% (G) | 0.001% | X | X |
| HUMIRON Fe 8% (G) | 0.1% | X | X |
| HUMIRON Fe 8% (G) | 0.2% | X | X |

X –application of nutrient solution.

The most deteriorating factor was air temperature during the night. Regarding that optimal temperature conditions for cucumber plants should not be lower then 24-25°C, it can be stated that test plants in this experiment were subjected to suboptimal growing conditions (Figure 4.7). The fluctuations of air temperature and relative air humidity are not optimal for the development of cucumber plants which in turn shortened vegetation time and plants productivity. Decrease of temperature during the night between 18.11.02 and 25.11.02 caused substantial damage to cucumber plants.

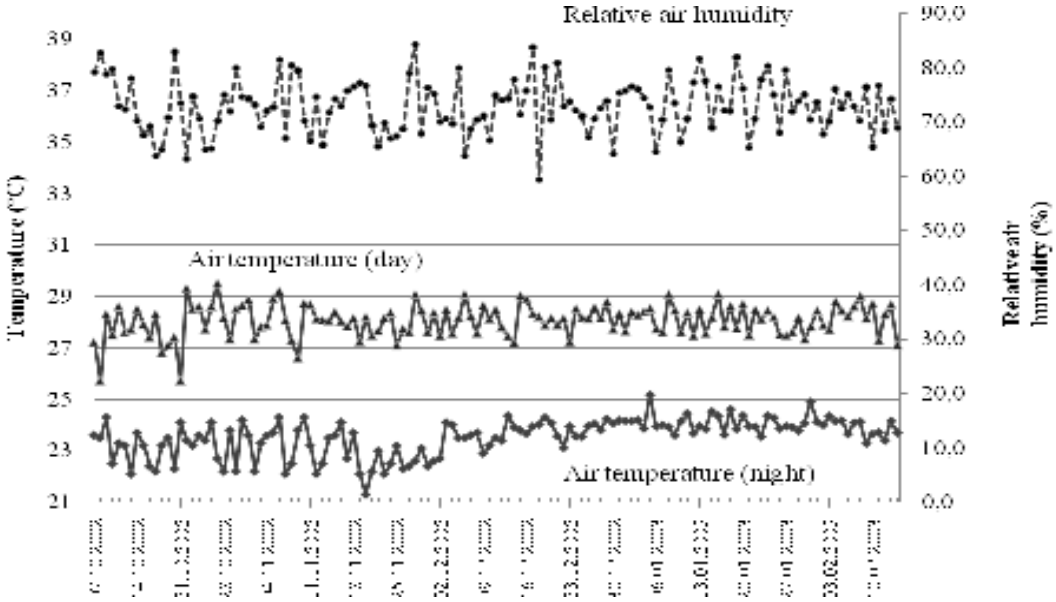


**Figure 4.7** Average temperature and relative air humidity recorded during experiment

Being sensitive to even mild climate changes, cucumber plants reacted to temperature fluctuations with lower growth rates. The temperature fluctuations shown in the figure 4.7 cannot be assessed as optimal for the growth and development of the cucumber plants. The difference between maximum and minimum temperature during the day in the experiment approaches 4°C. This difference is the same during the night, but absolute values of the temperature during the night is lower, so it can potentially damage the productivity of the cucumber plants. The same statement can be made in connection with air humidity in the experiment. The absolute difference between maximum and minimum values of the air humidity during vegetation is 40%. pH-reaction and conductivity of the nutrient solution were not stable during the entire vegetation period as it is shown in the figure 4.8. The most drastic changes in EC reaction of the nutrient solution occurred between 11.11.2000 and 18.11.2000.

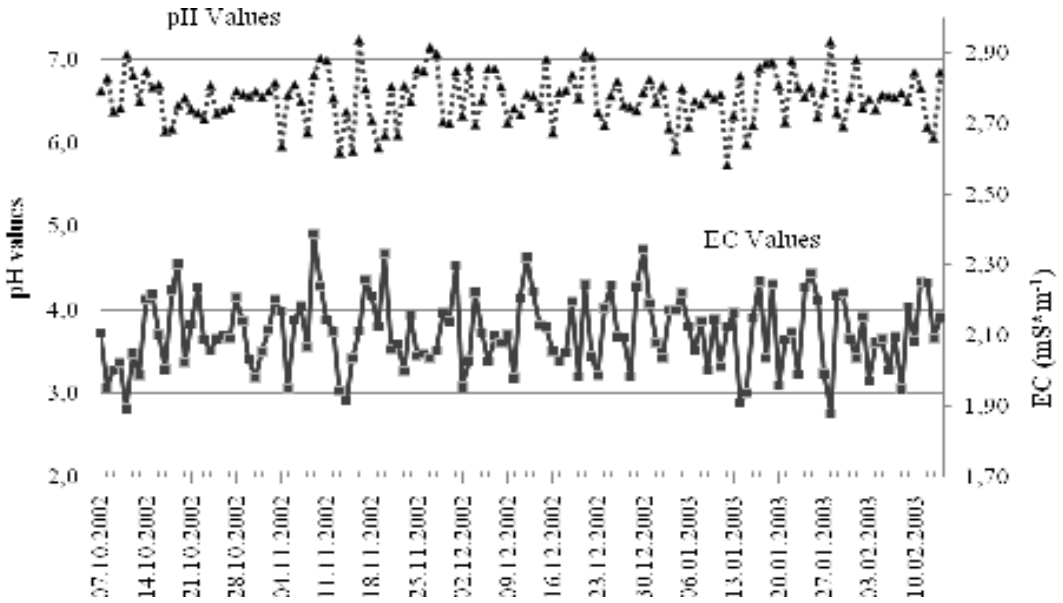


**Figure 4.8** Average pH and EC values of nutrient solution during vegetation period of cucumber plants

**Data Collection and Evaluation**

The growth and development of the experimental plants were recorded on the weekly basis. The major growing parameters recorded in this experiment were the leaf area and the height of the plants. These parameters were taken on the weekly basis. During the harvesting period, the cucumber fruits were picked according to the ripening. The first harvest of the cucumber fruits was discarded because it was collected before its full grown stage. The harvests for 10 days were pooled and evaluated together. The fresh weight of leaves and shoots was measured in the end of the vegetation.

Electric conductivity and pH-reaction of the nutrient solution were sampled and analyzed on the regular basis (weekly).

**Conditions for experiment: "Effects of humate, lactate and *Bacillus subtilis* on growth of cucumber plants". Subsection 5.1.2.**

The combination (mixture) of all these substances is aimed at widening of the activity spectrum in comparison to singular compound application. Combined biostimulating mixture, being applied several times at critical growth phases of the horticultural plants can contribute to the creation of the optimal growing conditions, as well as to mitigate adverse environmental factors, which are, as we can see from recorded data of pH, EC, air temperature and relative air humidity, very often going beyond boundaries of optimality. Characteristics of air condition (temperature

and relative air humidity) show substantial fluctuations. Relative air humidity through the experiment was unstable and recorded its maximum at 88.5% and minimum at 60% (Figure 4.9).

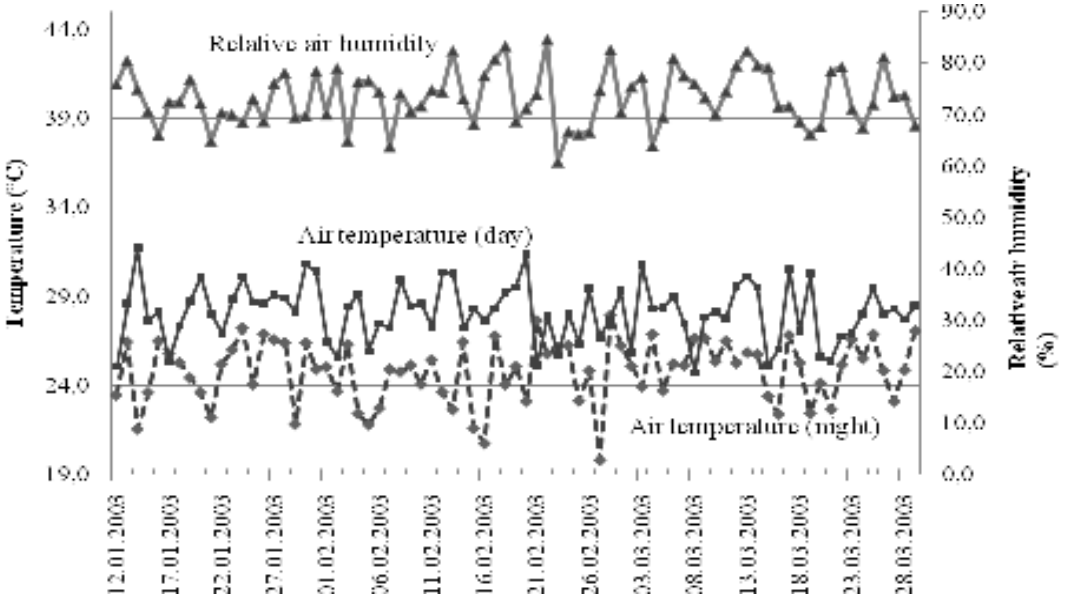


**Figure 4.9** Average air temperature and relative air humidity during the experiment

At the same time EC and pH values show substantial changes through vegetation period of cucumber plants (Figure 4.10). The absolute maximum value of the pH-reaction of the nutrient solution was 8. The absolute minimum value recorded in the experiment was 7.3. It can be stressed that these high values of the pH-reaction cannot be considered as optimal.

The dynamics of the EC-values during the experiment expresses an uneven pattern prone to drastic decreases 0.9 $mS*cm^{-1}$, on the 26.02.2003 as it is shown in the figure 4.10, and excessive increases up to 1.8 $mS*cm^{-1}$ on the 13.03.2003.

Application of biostimulating substances was achieved in two ways. The foliar application was conducted through spraying the solution of the respective substance or mixture of substances onto phyllosphere of the plant. The foliar application was compared to the rhizospheric application of the same substances.

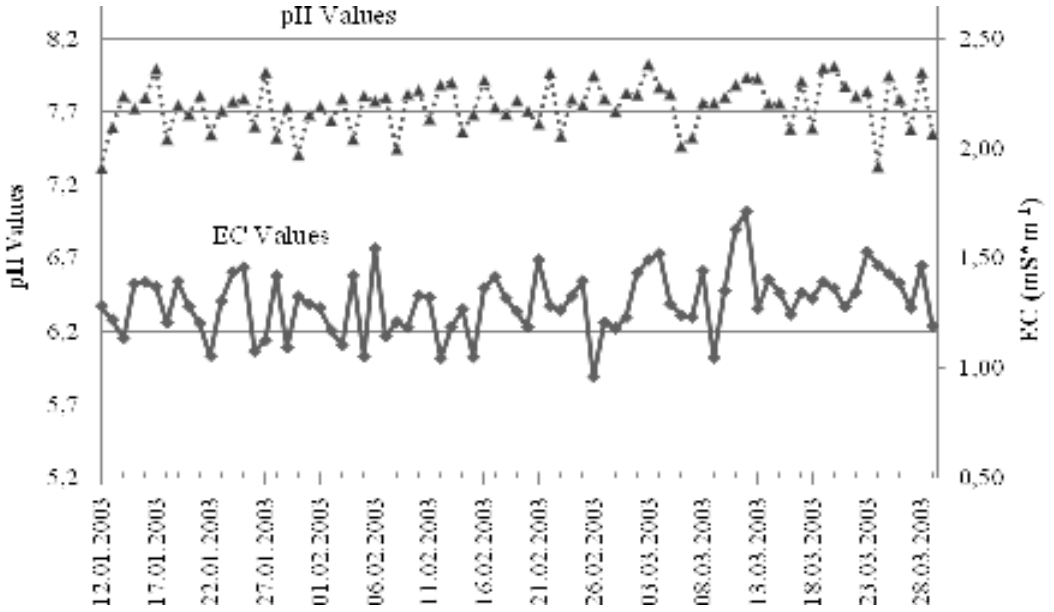


**Figure 4.10** Average pH and EC values of nutrient solution during vegetation period of cucumber plants.

The experiment is shown in the table 4.7 tests leaf and root application of LACTOFOL"O", HUMIRON(R), *B. subtilis FZB24* and combined biostimulating substance (HUMIRON Fe ®; Lactofol "O"; *B. subtilis FZB* 24®).

**Table 4.7** Scheme of the experiment

| **Leaf treatment** | | **Root treatment** | |
|---|---|---|---|
| **Variant** | **Concentration** | **Variant** | **Concentration** |
| Control | "-" | Control | "-" |
| Lactofol"O"® | 0.1% | Lactofol"O"® | 0.1% |
| HUMIRON Fe® | 0.001% | HUMIRON Fe® | 0.001% |
| *B. subtilis FZB* 24® | 0.2% | *B. subtilis FZB* 24® | 0.2% |
| Combined biostimulator (Lactofol"O"® + HUMIRON Fe® + *B. subtilis FZB* 24®) | All above mentioned concentrations are in effect | Combined biostimulator (Lactofol"O"® + HUMIRON Fe® + *B. subtilis FZB* 24®) | All above mentioned concentrations are in effect |

The experiment tests the effects of different applications of biostimulators and the influence of these factors on the photosynthetic apparatus and nutrition of the cucumber plants.

**Conditions for experiment: "Investigation of different forms of leaf treatments". Subsection 5.2.1**

**Growing conditions:** Light conditions, air temperature, relative air humidity. Average temperature during the day was equal to 28.3°C and during the night 23.1°C. The average

relative air humidity was recorded at 70%. Detailed description of growing conditions is in figure 4.11.

**Nutrient solution**: Standard nutrient solution. Plants were watered with 200ml of nutrient solution daily. For monitoring pH and EC values as well as nitrate content in nutrient solution a mix sample was prepared by sampling 100 ml of nutrient solution from every plant in the variants and mixing it together. After that 100ml of sample was taken for filtration with subsequent determination of pH, EC and $N\text{-}NO_3^-$.

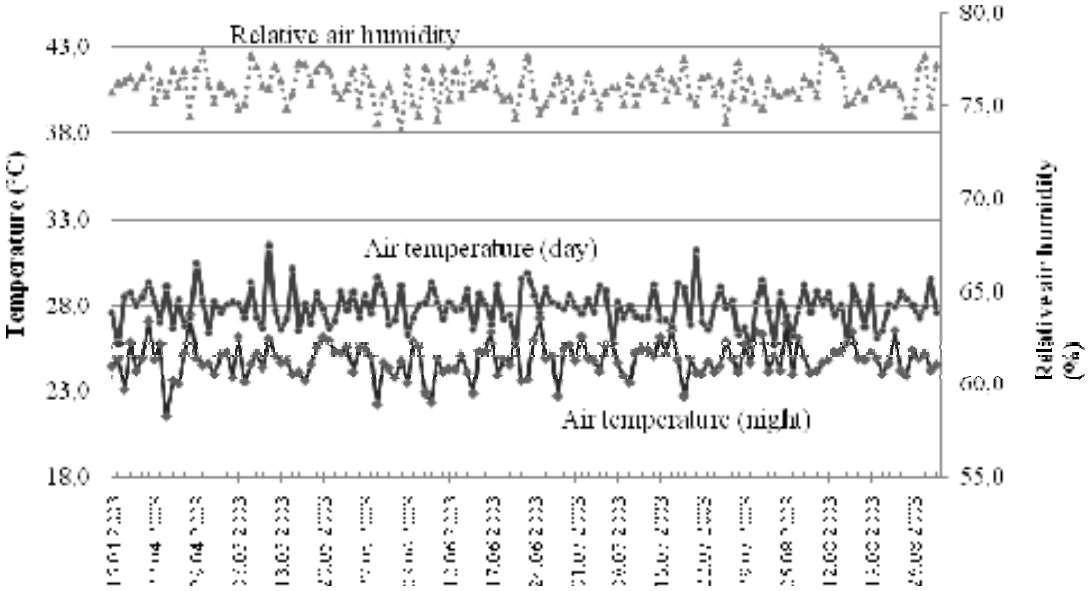


**Figure 4.11** Average day and night air temperature and relative air humidity during the experiment

Development of the air temperature during the day and night period of the vegetation was different. The night temperature was on average 4°C lower then the air temperature recorded during the day. The maximum temperature during the day was 30°C whilst the maximum temperature during the night was 27°C. The minimum values of the air temperature during the day and night periods were 25 and 20°C respectively. The data of pH and EC values during the vegetation period of cucumber plants are given in the figure 4.12.

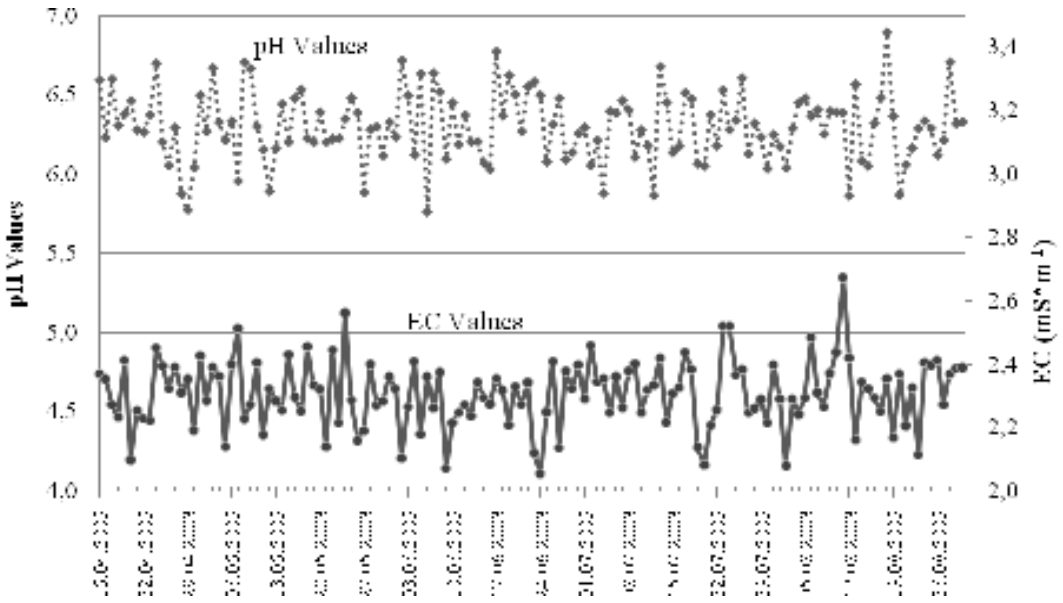


**Figure 4.12** Changes in pH-reaction EC of nutrient solution during vegetation

**Sowing**: The seeds of cucumber plant cv. Jassica (F1) were planted on 15-th of May 2003. Seeds were sowed in perlite substrate pending their germination. After germination on 10 of June 2003 plants were picked and transferred on rockwool bricks and on 25-th of June plants were planted in 8 liter Mitscherlich pots.

**Substrate:** Mitscherlich pots were filled with 8 l perlite. **Treatments:** 20 ml humate-solution was applied. Solution for leaf application with concentration 0.05% (w/v) HUMIRON-G® and K-humate was used. Plants were treated three times in weekly intervals in following development stages: first treatment: 5-6 leaves stage; second: 7-8 leaves stage; third: 9-10 leaves stage. Application of substances involved is performed through spraying of solution directly on plants. Research scheme is presented in the table 4.8.

**Table 4.8** Experiment scheme

| Variant | Concentration (%) | Quantity of substance applied (ml) | Treatment |
|---|---|---|---|
| **Control** | 0 | 0 | - |
| **Humiron (G)** | 0.05% | 20 | Upper surface of leaf |
| **Humiron (G)** | 0.05% | 20 | Lower surface of leaf |
| **K-Humate** | 0.05% | 20 | Upper surface of leaf |
| **K-Humate** | 0.05% | 20 | Lower surface of leaf |

**Conditions for experiment: "Investigation of plant biostimulators in different applications". Subsection 5.2.2**

The experiment plan with the aim to investigate an influence of the different applications of the biostimulators on the development of the cucumber plant is given in the table 4.9. The application of the biostimulators on the leaf surface is aimed at triggering responses at the level of the photosystem II. Contrary to the foliar application, the watering of the biostimulators or their mixtures, as it is shown in the table 4.9, to the root area of the plants can create more amiable conditions for the development of the test plants. This improvement in growing conditions may result from a balanced supply of the nutrients and formation of the malign microbial community within the plant rhizosphere influenced by the presence of *B.subtilis*.

**Table 4.9** Layout of the experiment

| Variant | Concentration | Application pattern | Quantity of solution |
|---|---|---|---|
| 1. **Control** | -* | - | Three times 20 ml plant$^{-1}$ |
| 2. **LACTOFOL** | 0.08% | Leaf application | |
| 3. **K-Humate** | 0.005% | Leaf application | |
| 4. *Bacillus subtilis FZB* 24® | Spore suspension (0.2%) | Leaf application | |
| 5. **LACTOFOL** | 0.08% | Watering in Substrate | |
| 6. **K-Humate** | 0.005% | Watering in Substrate | |
| 7. *Bacillus subtilis FZB* 24® | Spore suspension (0.2%) | Watering in Substrate | |
| 8. **LACTOFOL + K-Humate +** *Bacillus subtilis FZB* 24® **(Combined biostimulator)** | 0.08%+0.005%+0.2 % (respectively) | Watering in Substrate | |
| 9. **LACTOFOL + K-Humate +** *Bacillus subtilis FZB* 24® **(Combined biostimulator)** | 0.08%+0.005%+0.2 % (respectively) | Leaf application | |

*Variants without application

Each variant has 10 repetitions. 1 plant is 1 repetition.

**Substrate.** The containers were filled with 8 l perlite.

**Nutrient Solution:** Standard Nutrient Solution.

**Phenological parameters**. Plants growth was monitored through measuring following parameters: Plant's height – weekly, number of leaves – weekly, leaf area was calculated on the

basis of leaf's length and width, number of leaves was counted weekly on the same day with plant's height measurement. Values of pH, EC, N-$NO_3^-$ in substrate was read on weekly bases by sampling nutrient solution from each pot and preparing mix sample. From these mixed sample 100 ml of nutrient solution was taken and filtered to remove any organic or inorganic particles from the nutrient solution. Evaluation of harvest was conducted in order to determine marketable and nonmarketable fraction of yield. Major criteria for harvest evaluation are described in the table 4.3. The root samples were removed from the substrate and washed out from remnants of perlite. The roots were cut in pieces approximately 5cm long than their length was measured using root scanner "Comair".

**Conditions for experiment: "Use of biostimulating mixture in hydroponical substrate culture". (Subsection 5.3.1)**

Plants of *Cucumis sativus L. cv.* Jessica were used in the experiment. The overall design of the experiment is shown on figure 4.13. The objective of the experiment is to test the biostimulating mixture in a long term experiment, using most common horticultural substrates. For this purpose several different substrates were used to compare plants performance with and without treatments. The selection of the particular set of the substrates was conditioned by their abundance in the horticultural practice. Nevertheless, there are two variants in the experiment with a novel substrate – sheep wool. The sheep wool in this case a by product of the sheep husbandry and cannot be used for other useful purpose but to be accommodated to the needs of agricultural use. As it is shown in the figure 4.13, the experiment compares different horticultural substrates with and without treatment with biostimulating mixture.

Four fruit rotations were undertaken to test influence of K-Humate, LACTOFOL "O" and *B.subtilis* FZB24 on cucumber plants. Experiment consists of 10 variants. The first 5 variants are treated by the biostimulating mixture of *Bacillus subtilis* FZB 24® 0.2% + K-Humate 0.01% + LACTOFOL "O" ® 0.1% of cucumber plants and represented by five different horticultural substrates: perlite, rockwool, coir, peat, sheep wool.

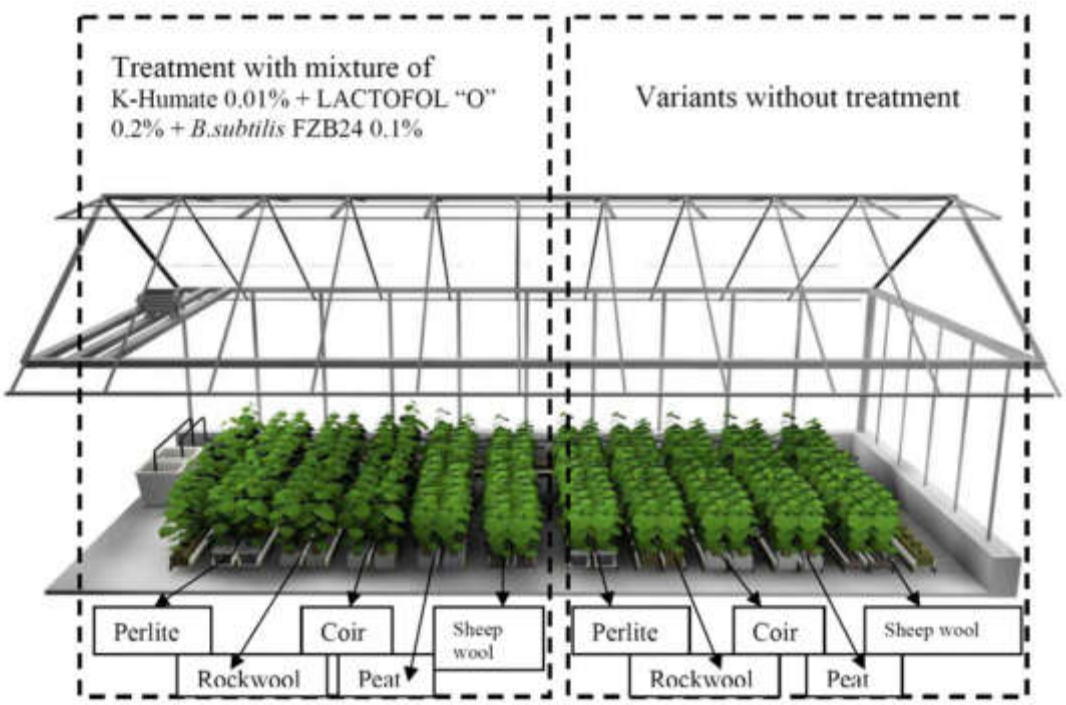


**Figure 4.13** Design of experiment for determination of long term use of cucumber plants and properties of horticultural substrates

The other half is the same combination of substrates but without treatment of the test plants. The growing conditions of the experiment are given in the table 4.10.

**Table 4.10** Growing conditions during the experiment

| **Parameter** | **Temperature °C** | | | **Nutrient solution** | | **Relative humidity** | **Solar radiation** |
|---|---|---|---|---|---|---|---|
| | Air | | Substrate | pH | EC | | |
| | *night* | *day* | | | mS * $cm^{-2}$ | % | KJ $cm^{-2}$ |
| **Rotation** | ***Maximal value (day) and minimal value (night)*** | | | ***Minimal and maximum value*** | | ***Mean values*** | |
| First rotation | 18.6 | 27.8 | 22.4 | 6.3-7.2 | 1.6-2.2 | 76.6 | 25 |
| Second rotation | 21.5 | 32.7 | 23.0 | 6.4-6.8 | 1.8-2.3 | 79.7 | 55 |
| Third rotation | 23.3 | 31.2 | 22.2 | 6.3-7.0 | 1.8-2.4 | 77.3 | 22 |
| Fourth rotation | 23.3 | 31.3 | 23.3 | 6.5-7.1 | 1.9-2.4 | 76.8 | 52 |

Plant density: 1.9 plants $m^{-2}$;
Plants are located on totally 137$m^{-2}$

The variations in solar radiation and air temperature data can be explained by the fact that the experiment was planned for the duration of two years, and different vegetation periods coincide

with different seasons of the year (table 4.10). Standard nutrient solution was used. Trickle irrigation was applied to deliver nutrient solution to plants 6 to 12 times a day 250ml per dripping cycle. Irrigation of nutrient solution was conducted with pauses of 12-15 min. Disease and pest control within experiment is depicted in the table 4.11.

**Table 4.11** Fruit rotations and pest control in the experiment.

| Operation | First rotation | Second rotation | Third rotation | Forth rotation |
|---|---|---|---|---|
| **Disease and pest control** | Masai+Plenum on 23.03.04 and 8.04.04 | Masai on 21.07.04 | Masai on 15.03.05 | Masai on 21.07.05 |

**Treatment**. Plants were treated with a mixture of *Bacillus subtilis FZB* 24® 0.2% + K-Humate 0.01% + LACTOFOL "O" ® 0.1%. Each component has a volume of 100 ml. Preparation of *B.subtilis FZB* 24® was conducted as follows: All components are mixed up before application. Timing of treatments with biostimulating mixture of *Bacillus subtilis FZB* 24® 0.2% + K-Humate 0.01% + LACTOFOL "O" ® 0.1%: 1-st: 5-6 leaves; 2-d: 6-7 leaves; 3-d: 7-8 leaves.
The research scheme of the experiment is shown in the table 4.12.

**Table 4.12** Research scheme of the experiment

| Variant | Vegetable | Quantity of plants | Replications | Treatment | Cultivar |
|---|---|---|---|---|---|
| 1. Perlite | Cucumber | 20 | 4 | Without treatment | Indira RZ (F1) |
| 2. Rockwool | | 20 | 4 | | |
| 3. Coir | | 20 | 4 | | |
| 4. Peat | | 20 | 4 | | |
| 5. Sheep wool | | 20 | 4 | | |
| 6. Perlite | | 20 | 4 | *Bacillus subtilis FZB* 24® 0.2% + K-Humate 0.01% + LACTOFOL "O" ® 0.1% | |
| 7. Rockwool | | 20 | 4 | | |
| 8. Coir | | 20 | 4 | | |
| 9. Peat | | 20 | 4 | | |
| 10. Sheep wool | | 20 | 4 | | |
| **Total** | | 200 | - | 3-times | - |

**Statistical analysis of the data**

Data were evaluated by ANOVA (Multifactor) to find differences between treatments. Means were compared using significant difference LSD test and the Chi-square-test (Pearson).

**Conditions for experiment: "Influence of the biostimulating mixture on the root length and biomass production". (Subsection 5.3.2)**

The fact that many horticultural substrates possess characteristics that hamper the process of the root system measurement, additional experiment was conducted. The objective of this experiment is to evaluate an influence of the biostimulating substances on the root system of the plant. Experiment was started to test plants reaction on treatments with K-Humate, LACTOFOL "O" *B.subtilis* FZB 24 and their combinations with regard to the root system development.

**Growing conditions.** Mean temperature during the day time was 27.8°C, night – 24.5°C, relative air humidity 75.2%. Cucumbers were grown in a substrate culture used containers with 9 liters perlite. Nutrient solution of standard composition was used. Trickle irrigation was applied to deliver nutrient solution to plants 6 to 7 times a day 230ml per dripping cycle. Irrigation of nutrient solution was conducted with pauses of 12-15 min. with trickle irrigation.

Experiment scheme:

1. Control variant
2. LACTOFOL "O" + *B.subtilis* FZB24;
3. K-Humate + *B.subtilis* FZB 24;
4. LACTOFOL "O" + *B.subtilis* FZB 24 + K-Humate.

**Harvesting.** Plants were harvested first time on 13.04.06. Fruits were sorted by variants according to the research scheme of the experiment. The harvested fruits were evaluated according to their weight, length and diameter. The appropriate class of the harvested cucumbers was assigned.

Evolution of climatic conditions within vegetation period of the experiment is shown on the figure 4.14. Air temperature recorded during the night and day manifests some drastic fluctuations. The maximum daily temperature reached 29.6°C, at the same time, the minimum temperature during the day was at the level of 26°C. Most troublesome development of the air temperature occurred during the tight time.

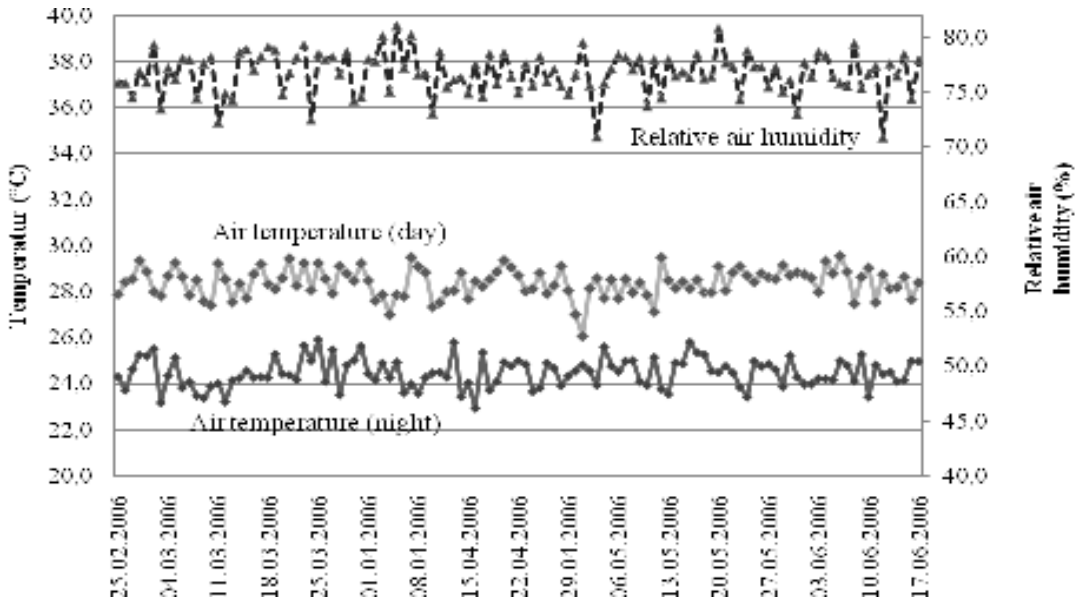


**Figure 4.14** Average temperature and relative air humidity recorded during experiment

Maximum night temperature reached the point of 26°C and the minimum one was recorded on the level of 23°C. Combined with information about air humidity where spread between maximum and minimum is 10% we can deduce that such temperature fluctuations are rather conducive for development of different diseases (mildew). On the other hand, instability of basic growing factors (due to the technical problems) can be considered as a limiting factor for cucumber productivity. pH values in their maximum of 7.2 and their minimum of 5.8 pose a real change for the test plants figure 4.15.

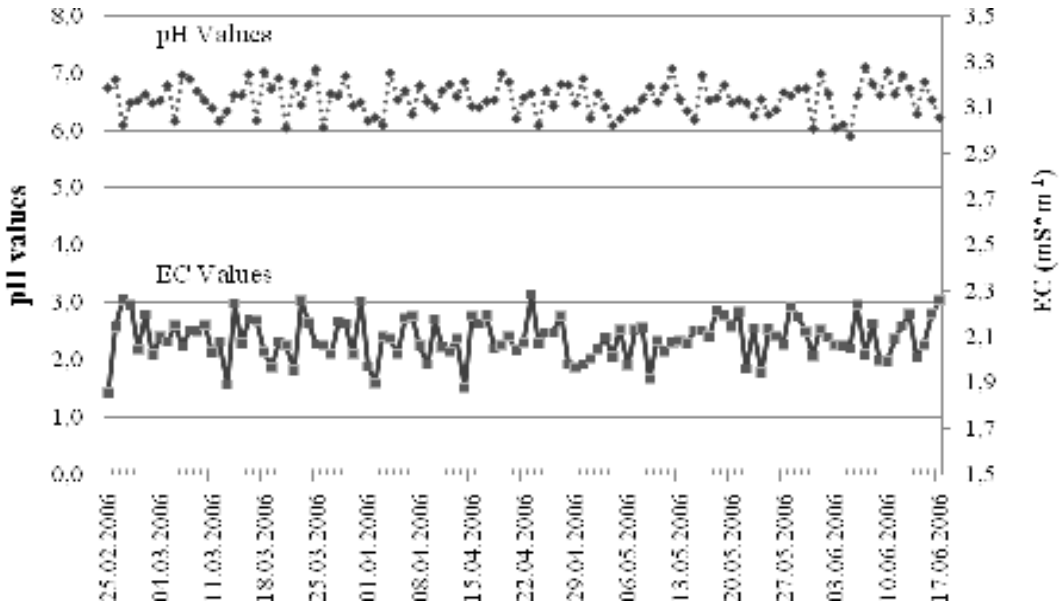


**Figure 4.15** Average pH and EC values of nutrient solution during vegetation period of cucumber plants

An optimal reaction of the nutrient solution changes between 5.9 and 6.5 depending on the development stage of the plant (DAVID *et al.,* 1994). Any other values out of this range can be taken as suboptimal ones. In this case, the root system of the cucumber plants is less capable of taking up the nutrient elements from the solution, which in turn aggravates their productivity.

**Conditions for experiment: "Effect of the biostimulating mixture under abiotic stress conditions". (Subsection 5.3.3)**

**Plant material and growing condition.** For current research we used plants of *Cucumis sativus L. cv.* Jessica. Cucumber seeds of cultivar Jessica for pH-stress experiment with alkali conditions were sowed on 5-th of August 2004 and transplanted to Mitscherlich pots on September 20-th 2004. Plants for experiment with suboptimal acidulous conditions were sown on 15-th of December 2004. Planting was conducted on 15-th of January 2005. The cucumber plants for temperature stress research were started on 9-th of February 2005 and transplanted into vegetation pots on 2-d of March 2005. Plants were watered with 250ml of nutrient solution daily. Plants were cultivated in climate chamber with adjustable temperature (25°C) and air humidity (80%). Plants were divided into two equal groups, first group was treated with biostimulating mixture (*Bacillus subtilis FZB* 24® 0.2% + K-Humate 0.01% + LACTOFOL "O" ® 0.1%) in amount of 300 ml once a week. Three treatments were conducted during three weeks with equal time lag between them (Table 4.13).

**Table 4.13** Research scheme of the experiment

| N/N | Treatment* | Concentration respectively | Number of test plants |
|---|---|---|---|
| 1 | **Control (without treatment)** | - | 4 |
| 2 | ***Bacillus subtilis FZB* 24® + K-Humate + LACTOFOL "O" ®** | 0.2%; 0.01%; 0.1% | 4 |

*treatment was conducted once a week three weeks in line.

Biostimulating mixture was watered into substrate. The application of the suboptimal growth factors is conducted at the end of the treatment phase. The air temperature in climate chamber was reduced from 25°C to 6°C with duration of 3 hours (Figure 4.16). After this phase, normal temperature conditions were restored.

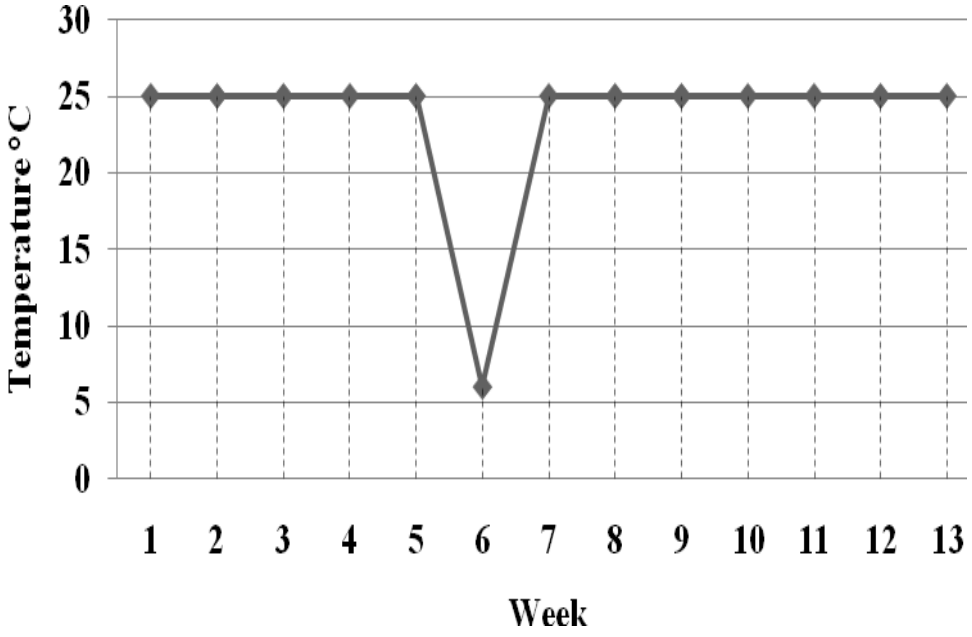


**Figure 4.16** Subnormal temperature conditions as stress factor

In the experiment with pH as suboptimal growth factor – pH values were adjusted to suboptimal level by adding several drops of $H_3PO_4$ to nutrient solution (assiduous conditions) (Figure 4.17). Alkali reaction of the nutrient solution was adjusted by adding several drops of KOH. To avoid variations in pH values between different plants $H_3PO_4$ and KOH were added to new nutrient solution of 10 liter volume, which in turn was divided between plants during all phase of suboptimal pH reaction. pH-stress was maintained through 1 week.

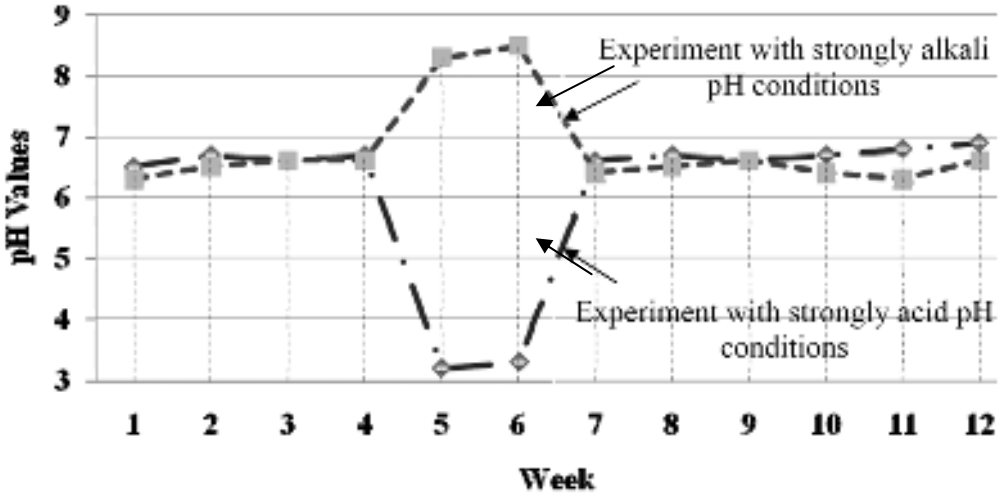


**Figure 4.17** Subnormal pH values applied as a stress factor

After one week nutrient solutions with suboptimal pH values were substituted with new nutrient solutions with pH reaction 6.3-6.7.

### 4.8.2 Physiological methods

**Chlorophyll-*a* fluorescence.** Measurements chlorophyll-*a* fluorescence was conducted with PAM-2000 (Fa. Walz). The main operational principle is a saturation pulse method on dark adapted plants. The dark adaptation of cucumber plants was achieved by wrapping the plant's leaves into aluminum folia to prevent incident of light on the leaf's surface (Figure **4.18a-b**). That causes the antennae of photosystem II to open, which in turn, creates a background for measurement of its electron efficiency. The dark adaptation was conducted during 30 min by reducing PAR (Photosynthetic Active Radiation) to the values equal or less then 10 µmol $m^{-2}s^{-1}$. The plant clip of the measuring device was attached to leaves surface and the measurement was conducted on the dark adapted plant. Three true leaves of every plant were selected to conduct chlorophyll-*a* fluorescence. 3 measurement points were allocated on every leaf. The measurement points were selected on the leaves representative for the given plant. In plants with 6-7 leaves three lower leaves were set aside for measurement. The cucumber plants on higher stages of their development often have multiple lateral shoots. In this case three well developed leaves from the top, omitting first 3-4 younger leaves, on the main shoot of the plant were selected. Collected chlorophyll-a fluorescence data were used to make inferences on electron efficiency of photosystem II of test plants.

Figure 4.18a Reading of chlorophyll-a fluorescence data


**Figure 4.18b** Process of dark adaptation of cucumber plants before chlorophyll-*a* florescence measurement

For interpretation of chlorophyll-*a* fluorescence, relationship Fv/Fm – which is also referred to as "Yield" that can be defined as electron efficiency of photosystem II (PSII) was used. The major criteria of electron efficiency of photosystem II is Fv/Fm value which in case of plants that are not under stress approaches value of 0.800. In those cases where Fv/Fm decreases below 0.770 and lower implies about stress situation in photosystem II.

### 4.8.3 Biological methods

**Microbial activity of the substrates.** Method of substrate-induced respiration is generally used in soil analysis. The method is based on the facts that soil associated microflora responds to introduction of glucose with immediate increase of respiration. The methodology was adopted for the horticultural substrates used in this experiment. The sample size was 400g from treated and non-treated variants. The initial preparation of the substrate samples involved removal of the coarse plant remnants. The substrate samples were frozen at -24°C and remained in such a state for 1 month pending an analysis. Before the experiment, the substrates samples were defrosted and the excess of water, leached from the substrates, discarded. The substrate samples, already at room temperature of 25°C were sieved through 10 mm sieve to remove any organic remnants of the plants. The first step of the experiment was to determine an optimal glucose concentration for subsequent analysis of substrate-induced respiration. Three different glucose concentrations were selected for the experiment (2, 3, and 4 $mg*g^{-1}$ of substrate). The research scheme is described in the table 4.14.

**Table 4.14** Research scheme for analysis of glucose induced and basal respiration of treated and non treated samples of substrates

| **Samples of Substrates** | | | |
|---|---|---|---|
| **Substrates with treatment** (K-Humate+LACTOFOL"O"+*B.subtilis* FZB 24) | | **Substrates without treatment** | |
| Glucose induced respiration | Basal respiration (without glucose addition) | Glucose induced respiration | Basal respiration (without glucose addition) |
| Determination of $CO_2$ concentration. | | | |

The entire experiment and the main operational steps within the experiment are shown in the figure 19. The step 1 describes the process of the sample taking, the step 2 explains the process of the sample fixation, the step 3 refers to the method of the sample analysis, and the step 4 addresses the processes of the data acquisition.

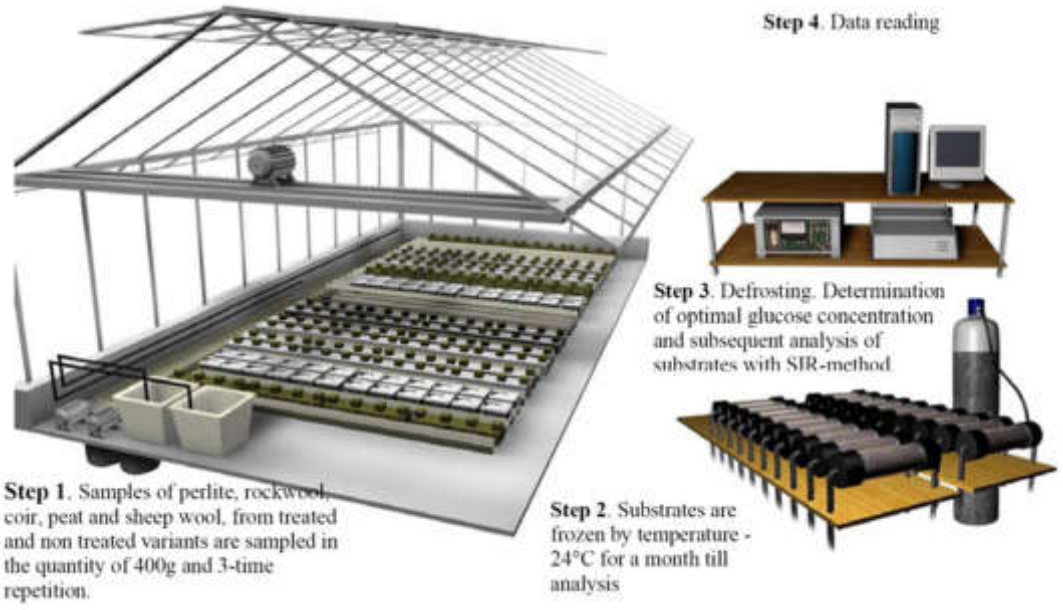


**Figure 4.19** Stages of sample taking, sample preparation and analysis of microbiological activity of horticultural substrates

A sample of 50g from every defrosted and sieved substrate was taken for determination of optimal glucose concentration. The optimal amount of glucose was determined by adding 2, 3 and 4 mg of glucose per every gram of substrate (mixed thoroughly) and subsequent determination of $CO_2$ release over 13 hour period. 4mg glucose $g^{-1}$ of the substrate was chosen as an optimal concentration – established in separate experiment. The substrates with addition of glucose were incubated for 13 hours. The data reading was conducted hourly, using ADC-225-

Mk3 fluorometer. Activity of microbial biomass corresponds to $CO_2$ outflow ($ml*h^{-1}$) after addition of glucose.

### 4.8.4 Statistical methods

**Statistics.** Data was tested for interdependency using Pearson correlation (SPSS) to test dependency between number of flowers and number of fruits. Standard error was estimated for root lengths of Cucumber plants. One-way ANOVA-test was conducted in one-factor experiments to analyze statistically significant differences in productivity of variants in experiments that showed pattern of normal distribution. Two-way ANOVA procedure was applied on experiments with normal distribution pattern as well as two-factor schemes. Data evaluated by ANOVA (SPSS) and the statistic tests Chi-square-test (Pearson) and Tukey-test. Data was tested for interdependency using Pearson correlation (SPSS). T-test was estimated for root lengths of cucumber plants. Integral $CO_2$ efflux was evaluated using ANOVA (SPSS) and the statistic tests Chi square (Pearson) and LSD.

# 5 Results and discussion

## 5.1 Effects of different concentrations and formulation of biostimulators

### 5.1.1 Effects of iron-humates on cucumber plants in substrate culture

**Problem description**

A string of positive effects caused by application of humates was detailed described in chapter 1. It was also specified that different humates extracted from different raw materials can have different characteristics. A raw material has an influence on relation between fulvate and humic of the humate, thus can lead to different effects when being applied to horticultural plants. Characterisation of disorders in the Fe-metabolism in horticultural plants can be connected with two situations: iron deficiency and iron toxicity.

IRON DEFICIENCY. The development of Fe-deficiency symptoms (iron chlorosis) is attributed to the alkaline nutritional conditions prevailing either in substrate or nutrient solution. $HCO_3^-$ and $NO_3^-$ have been identified as factors inducing Fe-deficiency chlorosis, as both compounds cause alkaline conditions in the apoplast of cucumbers after their take-over into plant metabolism (KIRKBY and RÖMHELD, 2004). Subsequently, the apoplastic pH rises and Fe is physiologically inactivated. The symptoms of iron deficiency in plants are chlorotic leaves. Often the veins remain green whereas the laminae are yellow, and a fine reticulate pattern develops with the darker green veins contrasting markedly with a lighter green or yellow background. Iron deficiency causes marked changes in the structure of chloroplasts (KIRKBY and RÖMHELD, 2004).

IRON TOXICITY. Iron toxicity is not a common problem in horticultural practice. Nevertheless an oversupply of mineral salt containing iron as a result either mismanagement or coincidence of growing conditions – regular flooding and drying of horticultural substrates results in gradual increase of mineral salts concentration around the root system of the plant. It can also occur in pot experiments. Visual indication of iron toxicity is that the whole leaf may turn brown, and the older leaves may die prematurely (BERGMANN, 1992). In some plants, leaves may become darker in colour and roots may turn brown (BERGMANN, 1992).

In this experiment different types of Fe-humate are tested. Figure 5.1 illustrates the influence of different iron-humates and their concentrations on the quantity of the leaves of cucumber plants. Application of Fe-humates extracted from both Russian and German raw material (Leonardite) proved to increase the quantity of leaves in comparison to variant without treatment (control).

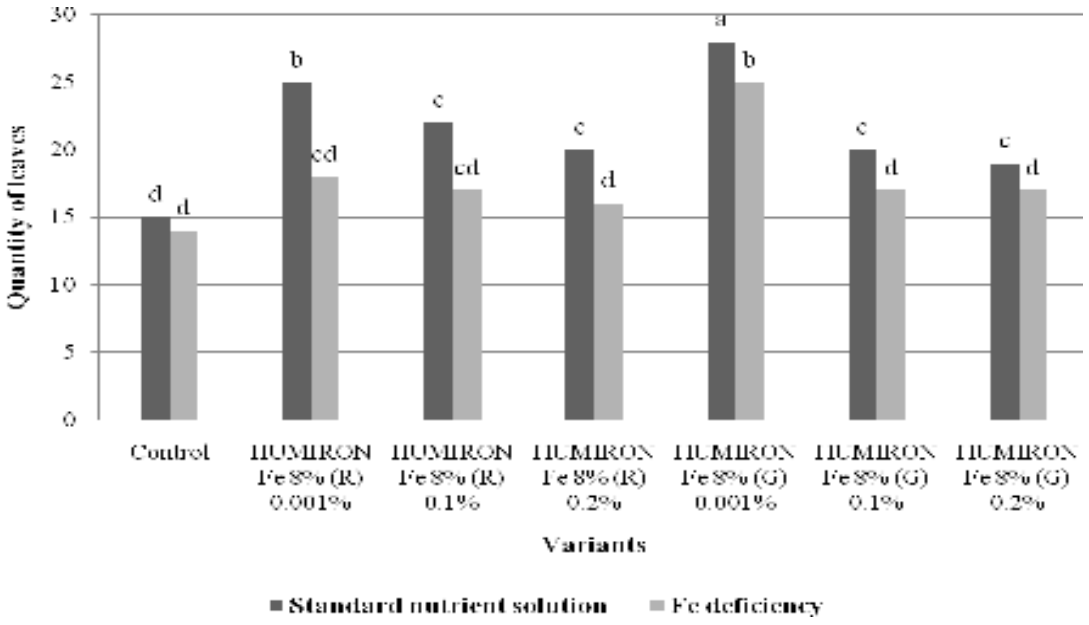


**Figure 5.1** Influence of HUMIRON (R) and (G) on leaf quantity at the end of the vegetation of cucumber plants. Two-way ANOVA. Tukey's HSD, $p<0.05$. Different letters indicate statistically significant difference. n=52

The control variant with and without Fe-deficiency did not show any statistically significant differences. The mean leaf quantity on the control with and without iron was 15 and 14 leaves respectively. Treatment with HUMIRON Fe 8% (R) in concentration of 0.001% proved to increase the leaf quantity on the cucumber plants to 25 leaves on the variant with standard nutrient solution and to 18 on the variant with iron deficiency. At the same time, application of HUMIRON Fe 8% (G) in the same concentration showed the highest significantly different result of 28 leaves on the variant with standard nutrient solution and 25 leaves with nutrient solution without iron. An effect of HUMIRON Fe 8% (R) on the variant with standard nutrient solution was the same as on the variants with HUMIRON Fe 8% (G) on the variant with iron deficiency. An increase in humate concentration to 0.1% and 0.2% inhibited the formation of leaves on both variants with and without iron deficiency.

Use of iron-humates had also different effects on leaf area of cucumber plants of the experiment (Figure 5.2). The lowest value of leaf area can be observed on the control variant with standard nutrient solution (2567 $cm^2$) and Fe-deficiency (2123 $cm^2$). Application of HUMIRON Fe 8% (R) in concentration 0.001% on the variant with standard nutrient solution was statistically significant higher in comparison to variants with concentrations of 0.1 and 0.2% of the humate derived from the same raw material (R), regardless of nutrient solution composition.

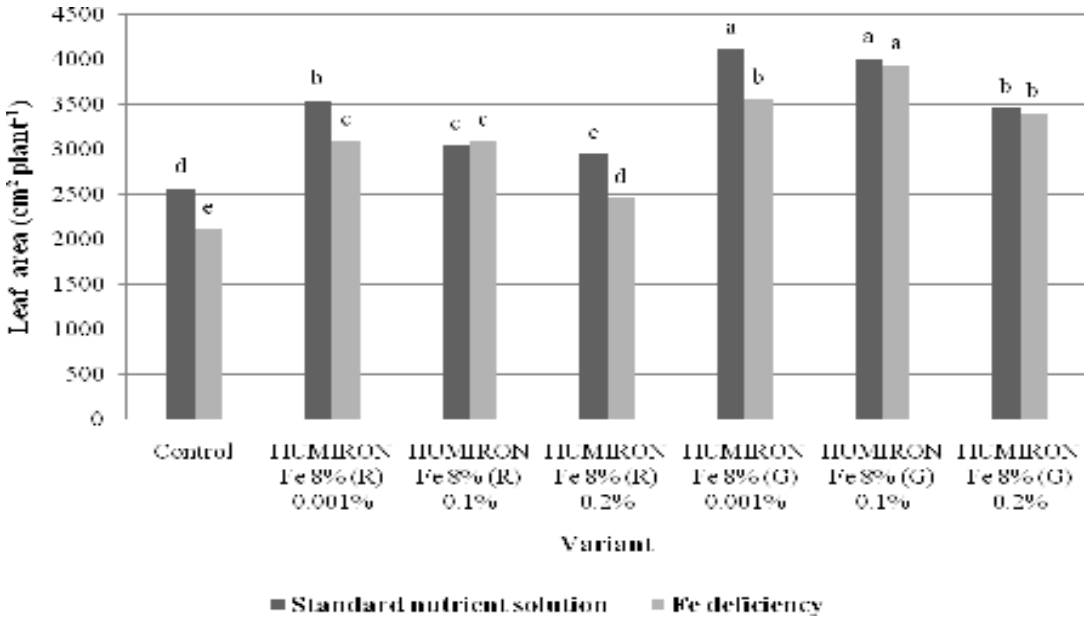


**Figure 5.2** Influence of HUMIRON (R) and (G) on leaf area of the cucumber plants at the end of vegetation of cucumber plants. Two-way ANOVA. Tukey's HSD, $p<0.05$. Different letters indicate statistically significant difference. n=52

Variant with HUMIRON Fe 8% (G) in concentrations 0.001% with standard nutrient solution had largest leaf area of 4120 $cm^2$ $plant^{-1}$ but at the same time this result is statistically significant only in comparison to the control variant and variants with humate extracted from Russian raw material. Statistically similar result was shown on the variant with the same iron-humate in concentration 0.1%. The leaf area on this variant with and without iron-deficiency in nutrient solution was 3945 $cm^2$ $plant^{-1}$ and 4012 $cm^2$ $plant^{-1}$ respectively. 10-fold increase in concentration of HUMIRON Fe 8% (G) to 0.2% showed decrease in leaf area – 3457$cm^2$ $plant^{-1}$ for standard nutrient solution and 3406 $cm^2$ $plant^{-1}$ iron-deficient nutrient solution.

On the whole, application of iron-humate has positive effect on cucumber plants on variants with different nutrient solutions. Use of iron-humate increases leaf count and leaf area of cucumber plant.

The harvest of cucumber plant and cucumber quantity in particular, was influenced by different concentrations of iron-humates of different origin (Figure 5.3). The highest fruit count was achieved on the variant with application of 0.1% of Fe-humate (G). The other concentrations of iron-humates extracted from different raw material inhibited formation of cucumber fruit. Their results were either on the level of the control variant or lower. Iron-deficiency in nutrient solution was the factor that limited formation of fruits on these variants, but at the same time,

only one variant with standard nutrient solution showed a positive development in fruit number – HUMIRON Fe 8% (G) 0.1%.

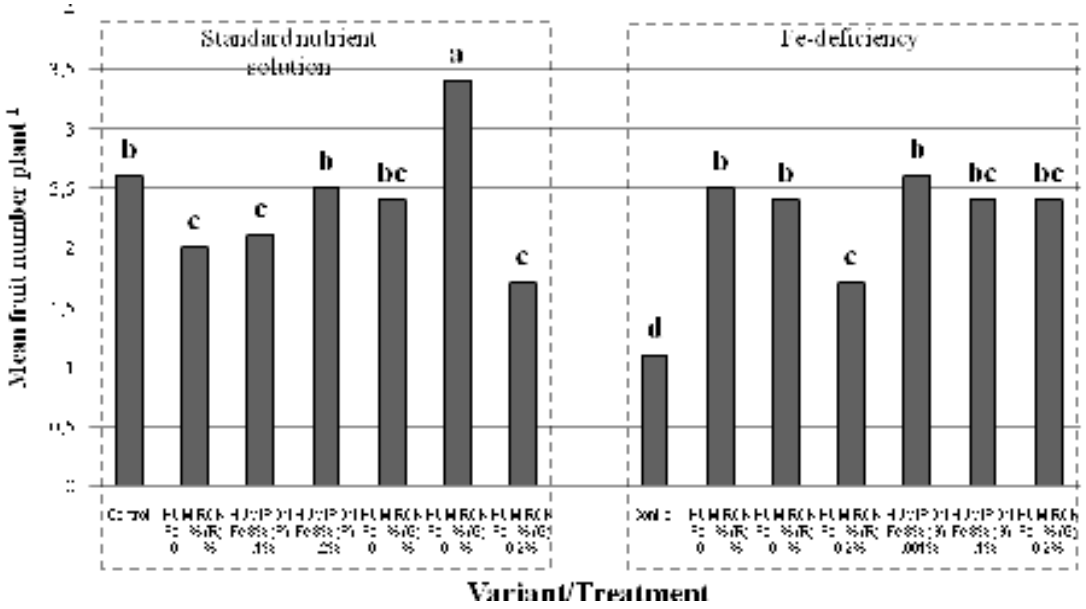


**Figure 5.3** Influence of HUMIRON (R) and (G) on fruit quantity of cucumber plants. Two-way ANOVA. Tukey's HSD, $p<0.05$. Different letters indicate statistically significant difference. n=52

Deficiency of iron in nutrient solution is a factor that decreases productivity of cucumber plants which is seen on control variants of both with and without iron nutrient solution.

The yield of cucumber fruits shows the same pattern as fruit number (Figure 5.4). Variants with Fe-deficiency were less prolific in terms of fruit yield. The highest yield achieved on the variant with application of HUMIRON Fe 8% (G) in concentration of 0.1%. Results from other variants with different concentrations of iron-humates with standard nutrient solution were lower then results on the control variant – without application of humates. The additive effect of iron-humate application, in this case, is a coherent positive effect on the cucumber plants as a result of both – standard solution and iron-humate application. Fruit yield formation of the cucumber plant depended on the availability of iron in nutrient solution. At the same time, application of Fe-humate on the variants with iron-deficient nutrient solution did not increase productivity of the cucumber plants.

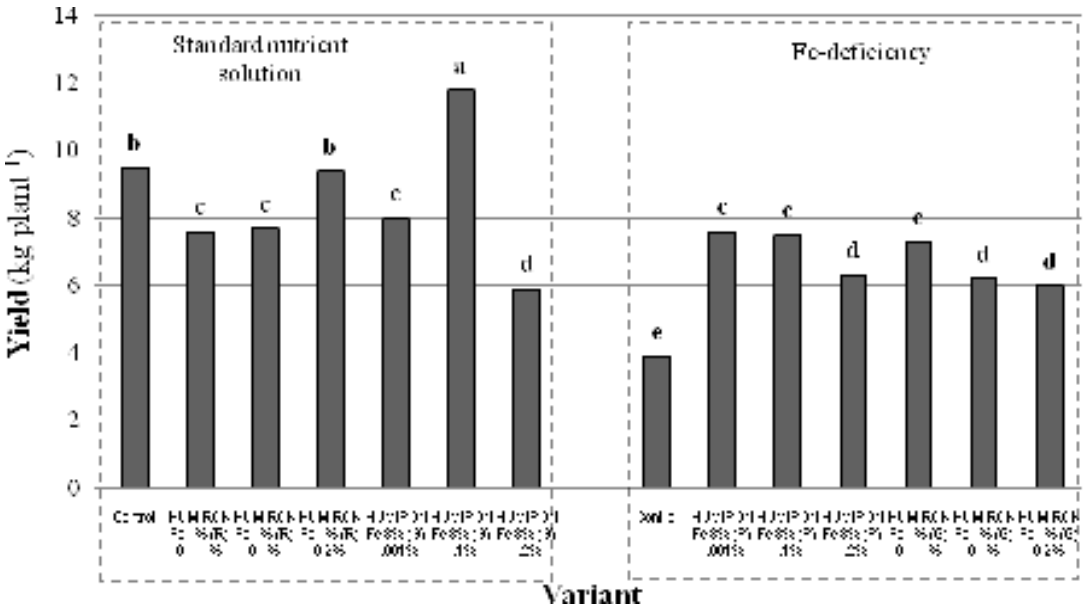


**Figure 5.4** Influence of HUMIRON (R) and (G) on productivity of cucumber plants. Two-way ANOVA. Tukey's HSD, $p<0.05$. Different letters indicate statistically significant difference. n=52

The effect that is observed on the variants with standard nutrient solution (Figure 5.4) can lead to the assumption that Fe-humates applied on the background of standard nutrient solution stimulates yield formation. Fe-humate extracted from German raw material with concentration 0.1% proved to be most effective in this experiment.

Several variants in the experiment with Fe-deficiency show statistically significant increase in fresh matter content of leaves. Only in one case of HUMIRON Fe 8% (R) 0.001% there is increase in fresh matter of the stems (Figure 5.5). An increase in concentration of HUMIRON Fe 8% (R) brought statistically significant increment in fresh matter of cucumber plants whilst application of HUMIRON Fe 8% (G) contributed to decrement of the fresh matter (Figure 5.5).

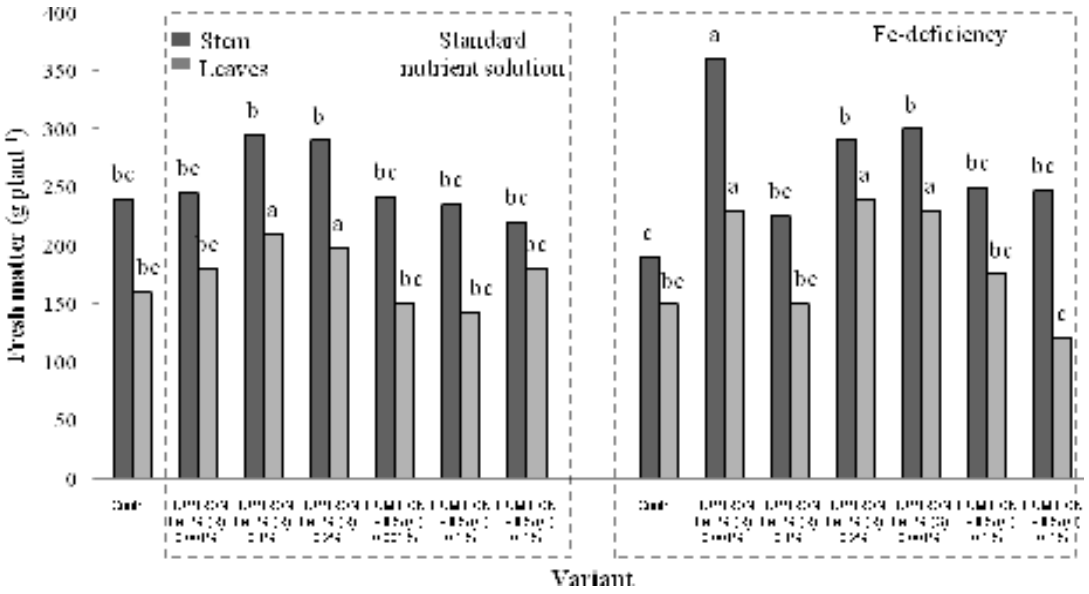


**Figure 5.5** Fresh matter of stems and leaves of cucumber plants. Statistical test conducted within variants with the same nutrient solution. Tukey's HSD, $p<0.05$. Different letters indicate statistically significant difference. n=52

Analysis of relation between leaf fresh matter and stem fresh matter of the cucumber plants (Figure 5.6) showed a weak relationship between these parameters. The cucumber plants cultivated in optimal growing conditions and without incidence of disease, unlike in this case, would show much closer correlation.

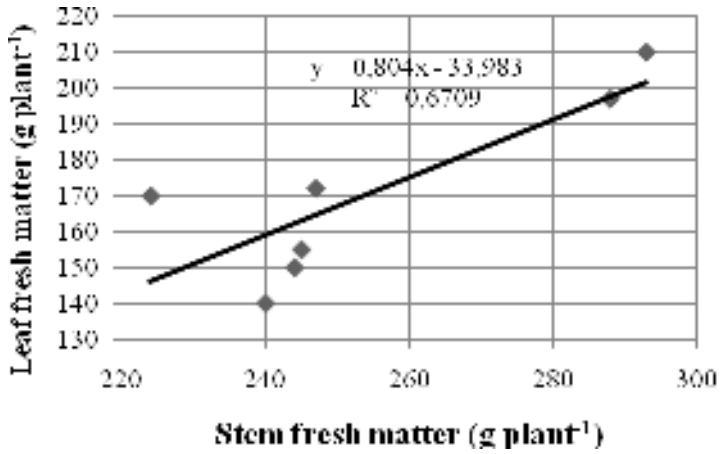


**Figure 5.6** Relation between leaf fresh matter and stem fresh matter of cucumber plants on variants with standard nutrient solution. Pearson correlation.

HUMIRON® can be used to improve plant growth and yield in substrate culture of cucumber. It is possible to apply HUMIRON® in the rhizosphere and on leaves as well. The influence of

humate shows statistically significant effects in experiments with Fe-deficiency and with standard nutrient solution. The effect was dependent on the concentration used and 0.2% HUMIRON® was inhibiting for yield. Relation between leaf fresh matter and stem fresh matter of the cucumber plants on variants with iron deficiency does not differ from that of variants with normal nutrient solution. There is a week connection between these parameters (Figure 5.7)

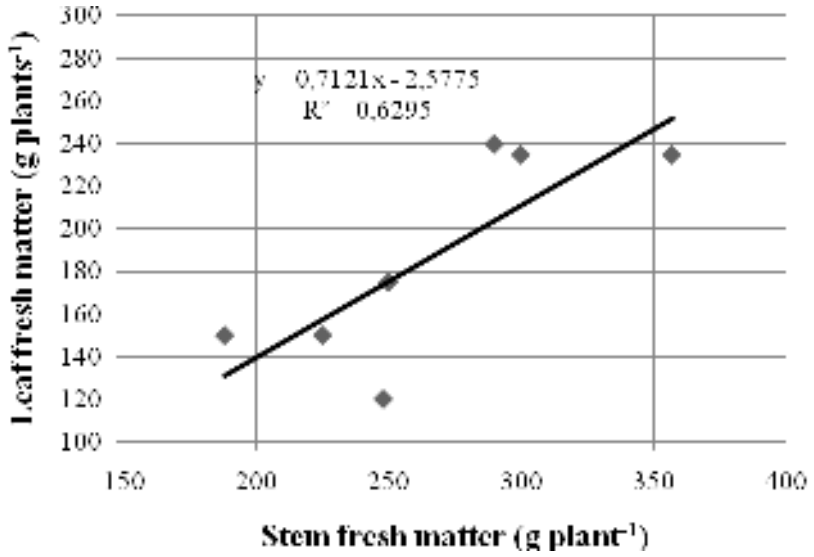


**Figure 5.7** Relation between leaf fresh matter and stem fresh matter of cucumber plants on variants with iron deficiency. Pearson's correlation.

The relations in figures 5.6 and 5.7 show the same correlation strength between leaf fresh matter and stem fresh matter from variants with and without iron deficiency. In general, such a correlation is a characteristic for plants that undergo or underwent substantial suboptimal growing condition(s). Combination of suboptimal growth conditions of both biotic and abiotic nature such as temperature and air humidity fluctuations, iron deficiency for some variants had a detrimental effect on development of cucumber plants. A drawn conclusion of this experiment can be that since there is no difference in correlations both variants are under stronger stress factor(s) then simple iron deficiency.

**Discussion**

Plants that can modify the rhizosphere to make iron more available can be classified as iron-efficient and those that cannot as iron-inefficient. It is among the iron-inefficient species that chlorosis is most commonly observed. Statistically significant differences in productivity of cucumber plants were registered on the variants with iron-deficiency in the nutrient solution. Application of iron humates, lead to the increase of plants productivity in comparison to the control variant. At the same time, on variants with standard nutrient solution we observe higher

productivity of plants on the three different variants including the control variant. Observed effects on the variants with standard nutrient solution can be explained by the additive influence of the iron-humate application and iron that is already available in the nutrient solution. On the variants with and without iron deficiency in nutrient solution the highest concentration of HUMIRON Fe 8% (R) brings the same result as the lowest of HUMIRON Fe 8% (G).

The highest fresh matter content of the cucumber shoots was observed on the variants with 0.001% of HUMIRON Fe 8% (R). The fresh matter of the leaves on these variants was not significantly different. The additional iron supply did not inhibit the growth and decrease the yield even if the iron supply in the nutrient solution is sufficient. The different effects of the humate types (HUMIRON R and G humate) compared here indicate that the influence of humate can be important in terms of treating acute deficiency of nutrient elements. On the other hand it cannot be sufficient in the long term perspective. Iron imbalances or deficiency can be counteracted by application of Fe-humate to the root zone. Combination of standard nutrient solution and HUMIRON Fe 8% (G) 0.1% creates best conditions for plants productivity.

### 5.1.2 Effects of humate, lactate and *Bacillus subtilis* on growth of cucumber plants

**Problem description**

Sustainable development of horticultural plants can be assured through application of different biologically active substances. In early experiments on the department of horticulture at Humboldt-University of Berlin it was shown that both foliar and root application can stabilize plant's system. At the same time, application of different biostimulators in the form of a biostimulating mixture (LACTOFOL"O"® + HUMIRON Fe(R) + *B. subtilis FZB* 24®) may have wider activity spectrum in comparison to there singular application. This can be explained by additive effects of all components present in the mixture. This effect can influence plants differently depending on how it is being applied. Foliar application through spraying is a way to influence development of photosynthetic apparatus of young plants as well as reduce deficiency of micronutrients in plantlets.

Phenological development of cucumber plants was influenced by the application of a mixture of all biostimulating substances can produce most beneficial effect on plants. This experiment tests this hypothesis. Foliar and root treatments using different biostimulators compared between each other (Table 5.1). The experiment tests foliar and root application of HUMIRON(R), *B.subtilis FZB24* and the combined biostimulating substance (LACTOFOL"O"; HUMIRON Fe(R); *B. subtilis FZB* 24®).

**Table 5.1** Variants and treatment of the experiment

| Leaf treatment | | Root treatment | |
|---|---|---|---|
| **Variant** | **Concentration** | **Variant** | **Concentration** |
| Control | "-" | Control | "-" |
| LACTOFOL"O"® | 0.1% | LACTOFOL"O"® | 0.1% |
| HUMIRON Fe(R) | 0.001% | HUMIRON Fe(R) | 0.001% |
| *B. subtilis FZB* 24® | 0.2% | *B. subtilis FZB* 24® | 0.2% |
| **Combined biostimulator** (LACTOFOL"O"® + HUMIRON Fe(R) + *B. subtilis FZB* 24®) | 0.1%+0.001%+0.2% | Combined biostimulator (LACTOFOL"O"® + HUMIRON Fe(R) + *B. subtilis FZB* 24®) | 0.1%+0.001%+0.2% |

HUMIRON Fe(R); LACTOFOL"O"; *Bacillus subtilis FZB* 24® were applied to define the influence on development of the photosynthetic apparatus, a leaf number and leaf area. As the experiment before showed the application of HUMIRON Fe(R) extracted from German raw material has better potential to improve plant's performance. At the same time it cannot be the case once it's being applied in the mixture with other biostimulators. As can be seen in figure 5.8, different treatments had significant effects. Leaf treatment of cucumber plants by LACTOFOL "O" brought results in terms of number of leaves of cucumber plants at the end of vegetation which is comparable with the same variant with root treatment.

Foliar and root treatment with HUMIRON (Fe-humate) of cucumber plants did not show any statistically significant differences. The same effect is observed on the variant with *B.subtilis* – leaf treatment. Being formulated to evolve its effect in the root area of the plant, *B.subtilis* showed statistically significant higher results on the variant with root treatment.

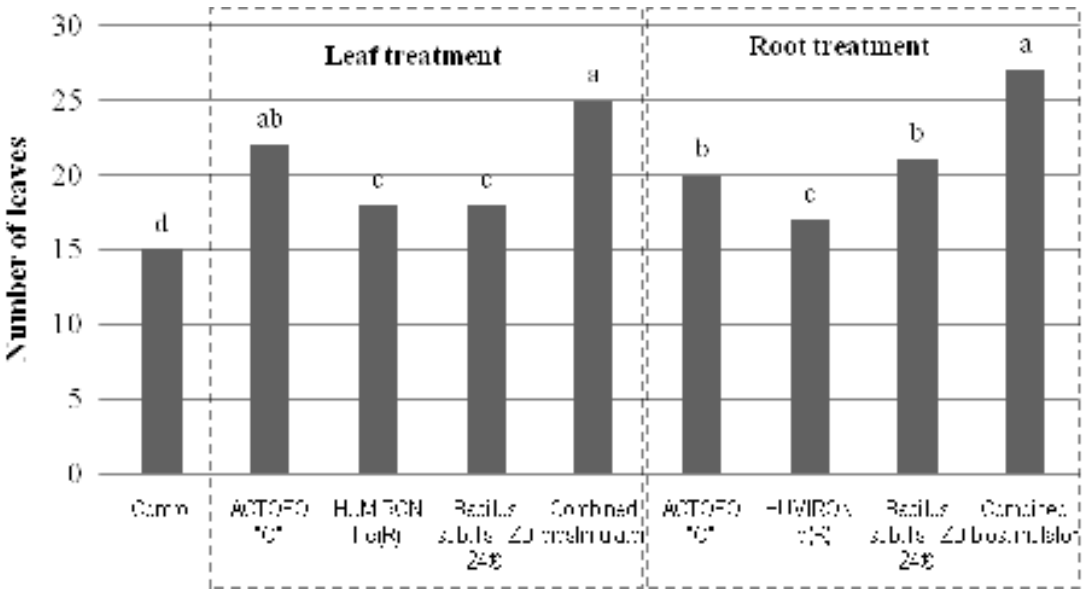


**Figure 5.8** Root and leaf application of biostimulators and their influence on the number of leaves on cucumber plants at the end of the vegetation. Two-way ANOVA. Tukey's HSD $p<0.05$. Different letters indicate statistically significant difference. n=36

Combination of all three substances (combined biostimulator) on both variants with foliar and root treatments showed highest results with 25 and 27 cucumber leaves respectively. There effect is seen as equal because of statistically insignificant difference between them. It is proved that all treatment had positive effects on cucumber plants with regard to leaf number count. Variant without treatments (Control) proved to have the lowest amount of leaves. At the same time, an inference can be made that three time application of biostimulating mixture in the form of LACTOFOL"O"® + HUMIRON Fe(R) + *B. subtilis FZB* 24® with concentrations 0.1%+0.001%+0,2% respectively and applied in amount of 20 ml showed maximum leaf count on both foliar and root treatment variants. Development of leaf area, however, showed different pattern (Figure 5.9). Application of LACTOFOL "O" on the variants with leaf and root application proved to have equal effect in terms of statistical significance. The same pattern showed on the variants with Fe-humate. Application of *B.subtilis* as leaf treatment had lesser effect (3120 $cm^2$) on the development of leaf area of the plants compared to the control variant (3567 $cm^2$). The same variant with root treatment showed statistically significant increment in leaf area (4500 $cm^2$). Application of a combined biostimulator for leaf treatment influenced development of leaf area (5230 $cm^2$) at the same level as root and leaf application of LACTOFOL"O" - 4788 $cm^2$ and 5337 $cm^2$ respectively.

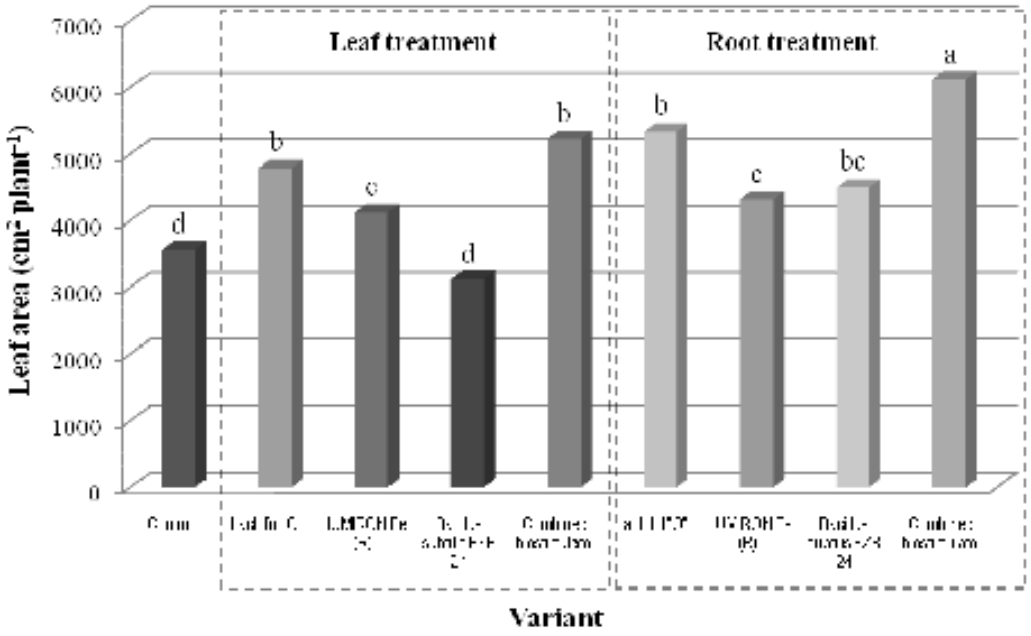


**Figure 5.9** Root and leaf application of biostimulators and their influence on the leaf area of cucumber plants at the end of the vegetation. Two-way ANOVA. Tukey's HSD $p<0.05$. Different letters indicate statistically significant difference. n= 36

The combined biostimulator on the variant with root application showed the highest result in terms of cucumber leaf area formation (6120 $cm^2$). This result is statistically significant in comparison to other variants of both leaf and root treatments. On the whole, the results point at the beneficial effects of LACTOFOL"O". Effects of LACTOFOL"O" in a concentration of 0.1% as foliar and root fertilizer induced second best results that are statistically insignificant only in comparison to combined biostimulator (foliar application). This fact suggest the possibility that the lactate plays the most important role as a constituent part of the combined biostimulator in increasing leaf area of the cucumber plants on leaf treated variants. At the same time, the role of lactate in the combined mixture applied into the root area is the same as *B.subtilis*. Formation of assimilation apparatus of the cucumber plants was statistically significant in variant with combined biostimulator applied into root area of the plants.

At the same time, plant's development is multipronged and the effects induced by utilization of different biostimulators are not confined to leaf formation. Figure 5.10 illustrates results of stem and root length of cucumber plants at the end of the vegetation. Treatment with LACTOFOL "O" as leaf fertilizer, did not show any statistically significant increase neither in stem nor in root

length in comparison to the control variant (without treatment). This effect was also observed in the variant with the lactate application in the form of watering to the substrate.

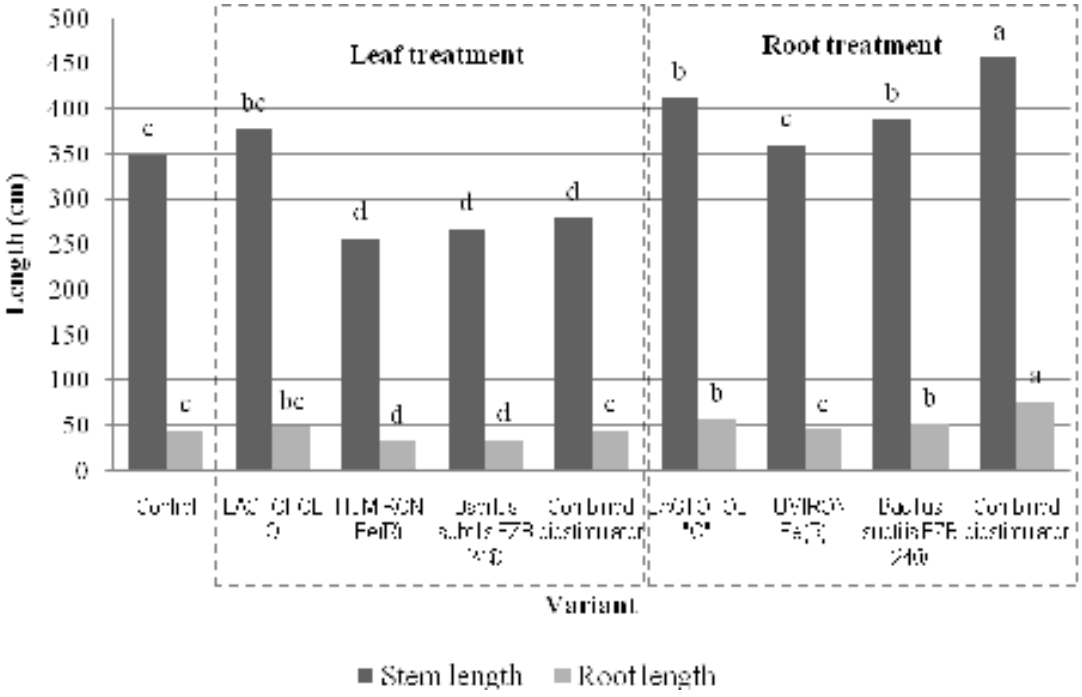


**Figure 5.10** Root and leaf application of biostimulators and their influence on the stem and root length of cucumber plants. Two-way ANOVA. Tukey's HSD $p<0.05$. Different letters indicate statistically significant difference. n=36

Use of HUMIRON Fe(R) showed statistically different results in the foliar and root application. Root length of cucumber plants on these variants was 32 cm in leaf application vs. 47 cm in case of root treatment. Stem length on the variants with leaf and root treatment was 256 and 359 cm respectively. Comparison between control and Fe-humate in leaf application showed that the use of iron-humate had an inhibiting effect on development of both stems and root length. Although watering of Fe-humate in the substrate in comparison to control variant did not have any statistically significant differences. Application of *B.subtilis* the phyllosphere of the plants did not show any increment of root and stem length of cucumber plants in comparison to the control variant. On the variant with the root treatment, however, it demonstrated an increase of root and stem length in comparison with variants with foliar treatments. Employment of combined biostimulator for leaf fertilization brought an inhibiting effect with regard to stem length development – 275 cm vs. 350 cm on control variant. The root length however was the same as in the variant without treatment. The same mixture being applied to the root system of the

cucumber plants showed the highest value in terms of stem and root length – 455 and 72 cm respectively.

In the process of vegetation, cucumber plants are forming regenerative organs and being parthenocarpic variety, flowers, subsequently forming cucumber fruits. During development as well as the ripening process of the cucumber fruits some flowers are falling out and some fruits do not ripen into the marketable fruit. Figure 5.11 illustrates two parameters: number of female flowers counted during vegetation and number of fruits harvested during vegetation. Leaf treatments by LACTOFOL"O", HUMIRON Fe (R) and *B.subtilis* had detrimental effect on flowers and fruits number of cucumber plants on the variants with leaf treatment.

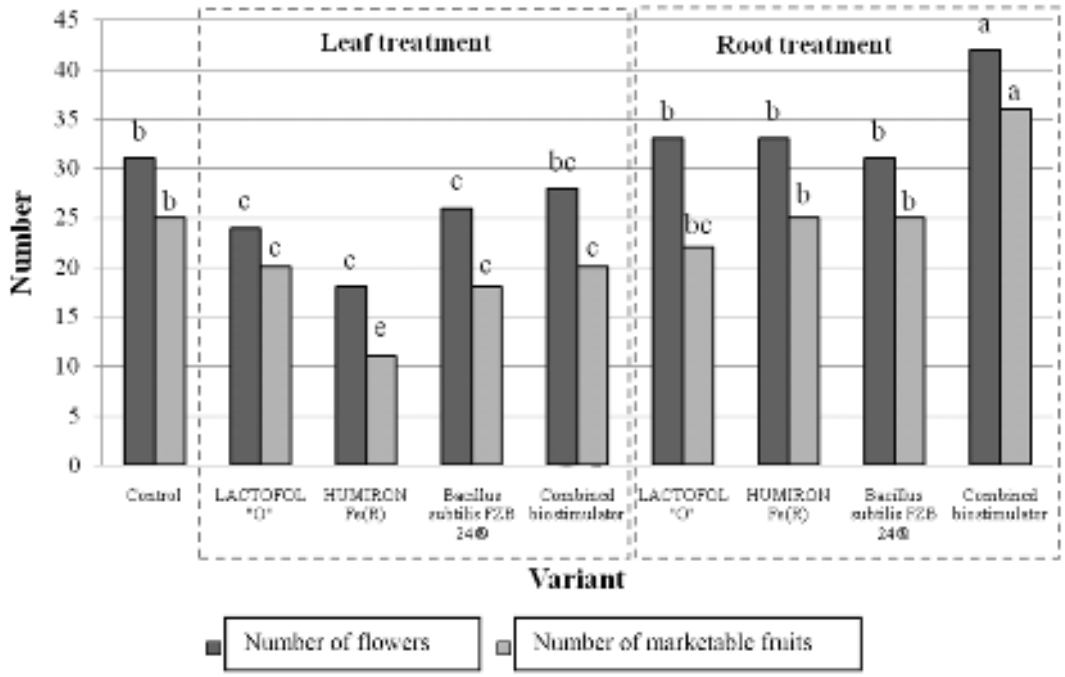


**Figure 5.11** Influence of different treatments on fruit quantity. Two-way ANOVA. Tukey's HSD $p<0.05$. Different letters indicate statistically significant difference. n= 36

Use of combined biostimulator in leaf treatment did not show any statistically significant difference in flower quantity - 28 in comparison to control and lower quantity of marketable fruits 20. The root treatment of the cucumber plants with combined biostimulator showed the highest results (Figure 5.11) in generation of flowers - 42 and that subsequently ripened into cucumber fruits - 36.

Joint use of three different biostimulators as a biostimulating mixture with general formulation LACTOFOL"O", humate and *B.subtilis* proved to be most effective once applied as root

fertilizer. Quantity of marketable and non-marketable fruits is shown in the figure 5.12. Use of lactate, humate and combined biostimulator in their singular application as leaf treatment proved to inhibit development of marketable fruit fraction fruit. Leaf treatment with *B.subtilis* brought the same yield of marketable cucumber fruits as the variant without treatment (control) 10.8 and 10.7 kg plant$^{-1}$ respectively.

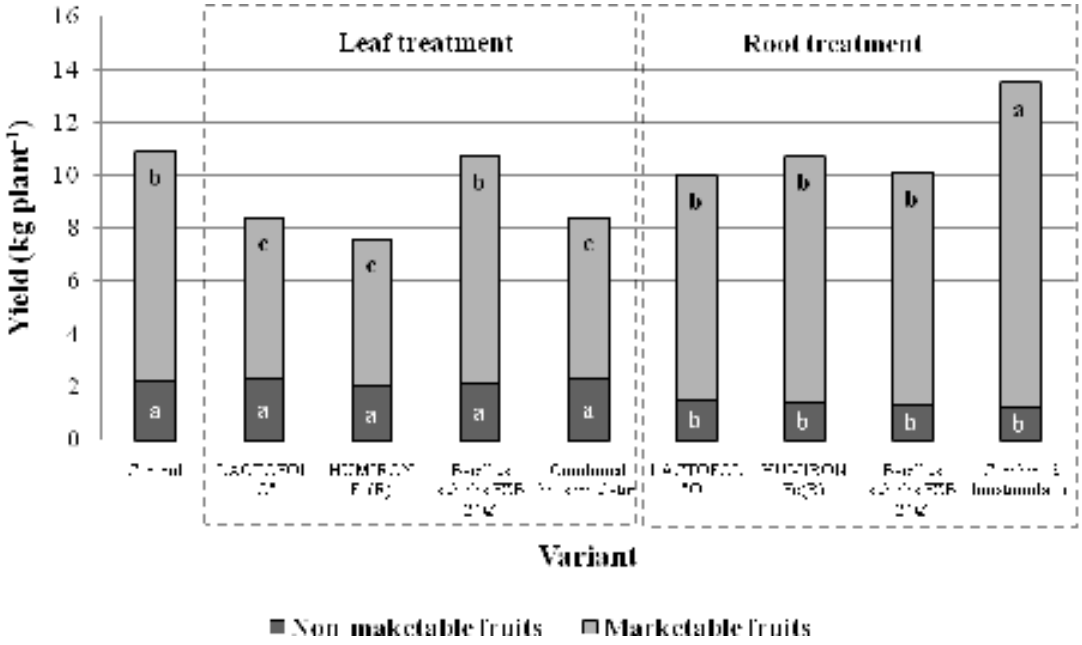


**Figure 5.12** Root and leaf application of biostimulators and their influence on the yield of cucumber plants. Tukey's HSD $p<0.05$. Different letters indicate statistically significant difference. n= 36

Variation in non-marketable fruit quantity of cucumber fruits with foliar treatment within all variants was not statistically significant. Root application of HUMIRON Fe (R); LACTOFOL"O"; *B. subtilis* FZB 24® exhibit no significant difference in productivity of cucumber plants on the control variant – without treatment. Singular application of biostimulators did not improve fruit quantity of marketable fraction on the variants with root treatment. However, fraction of non-marketable fruits proved to be lower in variants with the root treatments (Figure 5.12). The root treatments in the variant with combined biostimulator proved to be most effective in forming statistically significant maximum yield of the cucumber fruits. Although, quotient of non-marketable fruits on this variant differs statistically insignificant from data of other variants with the same treatment pattern, it showed an increase in cucumber fruit yield that is the highest in this experiment.

Being applied to the root system of the plants, combined biostimulator has a wider activity spectrum that is confirmed in the comparison with the other variants of this experiment. Physiological reactions of the plants adaptation to changing environmental conditions can be detected by measuring electron efficiency of photosystem II. The electron efficiency is expressed in terms of quantum yield of the photosystem II and shown on the figure 5.13.

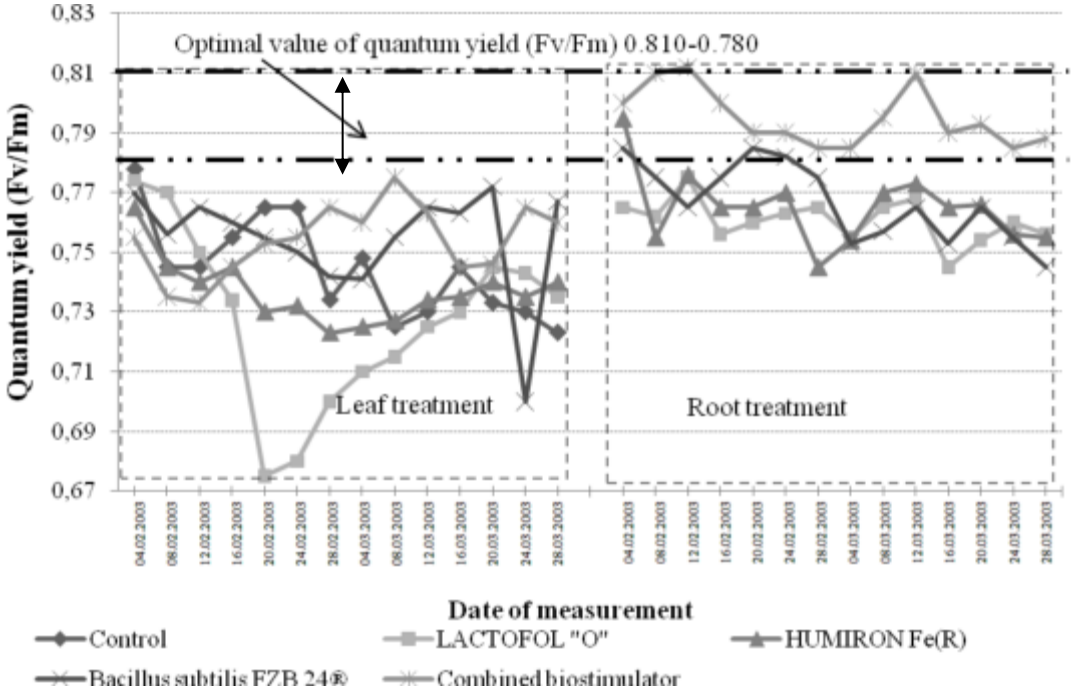


**Figure 5.13** Electron efficiency of photosystem II of the variants with different treatments. Statistics - repeated measures t-test $t(55) = -9.32$; $p<0.05$.

Optimal Fv/Fm value, according to different literature sources, is between 0.810 and 0.780. The values of quantum yield of assimilation apparatus of cucumber plants on the control variant and the other variants with leaf treatments are below optimal. The worst evolution of Fv/Fm is recorded on the variant with lactate used in form of leaf fertilizer (Figure 5.13). At the same time, electron efficiency of other variants with leaf treatment has never reached the optimal interval between 0.810 and 0.780.

Variants with root treatments showed better results with regard to electron efficiency of photosystem II, at the same time, only three variants (HUMIRON, *B.subtilis* and combined biostimulator) recorded at least during one measurement, values that coincide with the optimal range. The variant with combined biostimulator applied in the form of root treatment had the best result of Fv/Fm fluctuation of which was within the range of optimal value during all vegetation period.

**Discussion**

Application of LACTOFOL"O", HUMIRON Fe(R), *B. subtilis FZB* 24® as foliar and root fertilizer can positively influence development of cucumber plants. At the same time, their influence did not evolve in the same manner – due to different nature of these substances.

LACTOFOL"O" – salt of lactic acid saturated with micro- and macronutrients, is designed as foliar fertilizer. Being applied in the form of the leaf treatment it increases leaf area of the plants in comparison to control variants (Figure 5.9). Lactate increases elongation of root and stem of cucumber plants (Figure 5.10). Application of lactate as root fertilizer showed the same effects as in the variants with the foliar treatments.

Fe-humate involved in the leaf treatments did not show the same effects described with application of lactate. In fact, its application by spraying it on leaves of the cucumber plants negatively influenced their development. Use of Fe-humate may have positive results by its application in the substrate. It can be explained by the fact that humates tend to improve root growth of plants. Application of combined biostimulator by watering into substrate can lead to assumption that activity spectrum of combined biostimulator that consists of HUMIRON Fe8% (R); LACTOFOL"O"; *B. subtilis FZB* 24®. Cucumber plants yield formation is influenced by many factor of the growing environment. Formation of cucumber fruit yield of on the variants within this experiment can be characterized in terms of physiological reactions on the photosynthetic level. Exposure to different biotic and abiotic environmental factors may negatively influence plants productivity, which can be explained by reduction photosynthetic activity of the plants. Optimal value of quantum yield (Fv/Fm) generally varies between 0.780 and 0.810. Different treatment may induce higher of photosynthetic capability that finds its expression in chlorophyll-*a* fluorescence. At the same time, some forms of treatment may hamper or even inhibit development of photosynthetic apparatus.

## 5.2 Effects of different biostimulators as leaf and root application

Foliar application of leaf fertilizers is usually confronted by one equation – which is the best way of application – on the lower side of the leaf or on the upper one? Lower and upper epidermis is covered by hydrophobic cuticle. It covers the surfaces of the plant's leaf to reduce desiccation. The cuticle layer that covers the leaf surface is a very effective barrier to water movement. It has been estimated that only about 5% of the water is lost by diffusion of water vapor through the tiny pores of the stomatal apparatus, which are usually most abundant on the lower surface of the leaf. Applying foliar fertilizer to the lower part of the leaf can lead to better sorption of the

nutrients into the plant system. Vascular tissue is usually closer to the lower side of the leaf which makes possible to influence bioprocesses within the plant's leaf much quicker as in the case of root treatment.

### 5.2.1 Investigation of different forms of leaf treatments

**Problem description**

As it has already been shown in the previous experiments, leaf application of biostimulating substances do not always contribute to positive outcome in terms of plants' productivity. The major reason for the debacle of previous endeavors of leaf treatments is attributed to the fact that the application of biostimulating substance itself was fulfilled by spraying the solution upper surface of the plant's leaf. The iron-humate application in the form of foliar treatments under conditions with high air temperature can result in situations when the cell membranes are damaged and the biochemical reactions of the photosynthesis are slowed or stopped. Mismanagement in foliar application of plant nutrients, can severely reduce crop yields and makes plants more susceptible to diseases and insects. There are two major approaches in foliar fertilization: application of foliar fertilizer on the upper surface of the leaf, and on the lower one.

Upper leaf surface treatment

This is the easiest way of foliar fertilizer use. At the same time, in case of humate application it can lead to reduction of photosynthetic activity of leaves. The reason - upper leaf surface is directly exposed to incident photosynthetic active radiation (PAR). Humates, being very intense colored substances can block an income of photosynthetic active radiation, effectively hampering the day phase of the photosynthesis.

Lower leaf surface treatment

Macro- or micronutrients can enter the plants through stomata, tiny pores used by leaves for gas exchange. The question is: which area of the leaf is better for application of iron-humate? The fact that most stomata are located on the lower part of the plant's leaf leads to assumption that application of humates on the lower surface of the leaf can have better effects for biosynthesis and as a result –fruit formation of cucumber plants.

To investigate influence of Humates (K-Humate and Humiron (G)- iron-humate) on growth and development of cucumber plant (*Cucumis sativum L.*) cultivar Jassica (F1), and specifically on development of leaf area of plants. This study compares different foliar treatments and there potential effects on the development of cucumber plants. Leaf number at the end of the vegetation is shown in figure 5.14.

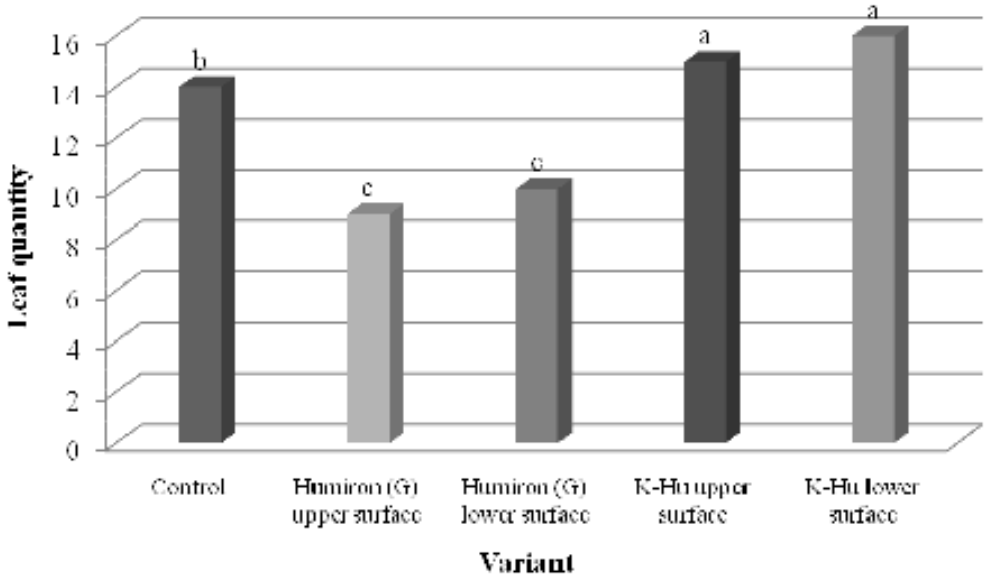


**Figure 5.14** Influence of different leaf treatment on leaf quantity of cucumber plant. One-way ANOVA. Tukey's HSD $p<0.05$. Different letters mean statistical significant difference. n= 20

Leaf quantity development on the control variant recorded at the end of the vegetation on average 13.8. Application of HUMIRON (G) in different treatments – upper and lower surfaces inhibited the development of leaves on these variants in comparison to the control and K-Humate variants. Leaf count on the variants with K-Humate applied on the upper surface and the lower surface do not express any statistically significant difference between each other. The leaf number on the variant with potassium humate applied on the upper surface is 14.5 and lower surface 15.6.

Being applied on the upper surface of the cucumber leaf, potassium-humate contributed to larger leaf area of the cucumber plants (Figure 5.15). The other treatments did not have statistically significant effect on the morphology of the leaves.

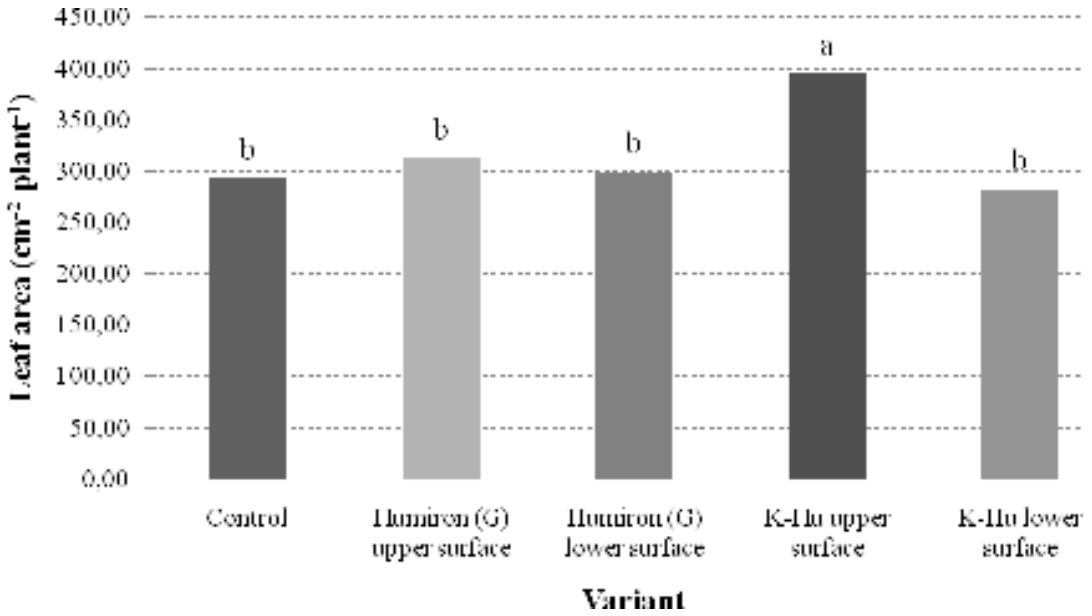


**Figure 5.15** Influence of humate treatments on leaf area of cucumber plants. One-way ANOVA.Tukey's HSD $p<0.05$. Different letters mean statistical significant difference. n= 20

The control variant showed the value of leaf area of 295 $cm^2$ $plant^{-1}$ when application of Fe-humate (HUMIRON (G)) on the upper surface of the leaf did not change its leaf area statistically significant and read 308.15 $cm^2$ $plant^{-1}$. The same pattern pertained for variants with HUMIRON (G) and potassium humate applied on the lower surface of the leaf – 300 and 281.12$cm^2$ $plant^{-1}$ respectively.

Influence of HUMIRON Fe 8% (G) and K-Humate had statistically significant effect on plants length (Figure 5.16). The variant without treatment – control has an average stem length of 211.12 cm, at the same time, the root length is on average 42 cm. Next variant with Fe-humate (G) used on upper surface of cucumber leaf contributed to elongation of both stem – 247.23 cm and root – 48,2 cm. These results in comparison to control variant represent a statistically significant increase in root and stem length. Variants with the same biostimulator being applied on the lower surface of the leaf showed on average the same results as on the variant with upper surface treatment.

Application of K-Humate contributed to the increase of the plants length on the variant with upper surface treatment – 275.21cm – a statistically significant increment of plant's length. The root length on this variant showed fluctuations in its values within the repetitions. That fact contributed to statistically insignificant result, in comparison to all previous variants.

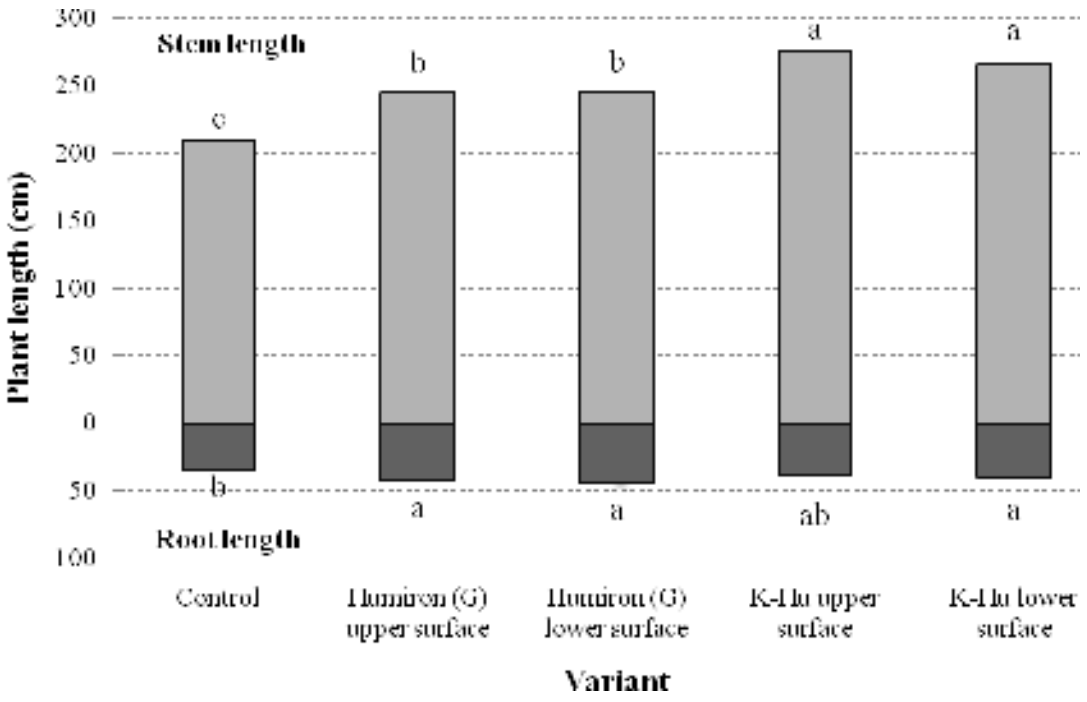


**Figure 5.16** Influence of humate treatments on plant length of cucumber plants. One-way ANOVA. Tukey's HSD $p<0.05$. Different letters mean statistical significance. n=20

Application of K-Humate on the lower surface of the leaf gave no statistically significant difference in root length in comparison with other variants.

Leaf treatments are only possible in combination with cultivars resistant to mildew. Otherwise fluctuations in relative air humidity and temperature in the juncture with the leaf treatment might contribute to the outbreak of the mildew. In all experiments where leaf treatments were undertaken, the problem of diseases may arise. At the same time, treatments proved ineffective in stimulating productivity of plants. Humate treatments did brought comparable results of plants' growth and productivity but at the same time it fell short of expected standards that are common in industrial practices where productivity of cucumber plants should not be less then 15 kg plant$^{-1}$. It brings an assumption that leaf application of humates alone cannot be used in stabilizing functionality of plant's system.

Considering the yield of the cucumber fruits in this experiment (Figure 5.17), we can conclude that application of potassium humate to the lower surface of the plant's leaf brought statistically significant increase in the yield of marketable fruits. At the same time, non marketable fruits were on the comparable level with the other variants except the control.

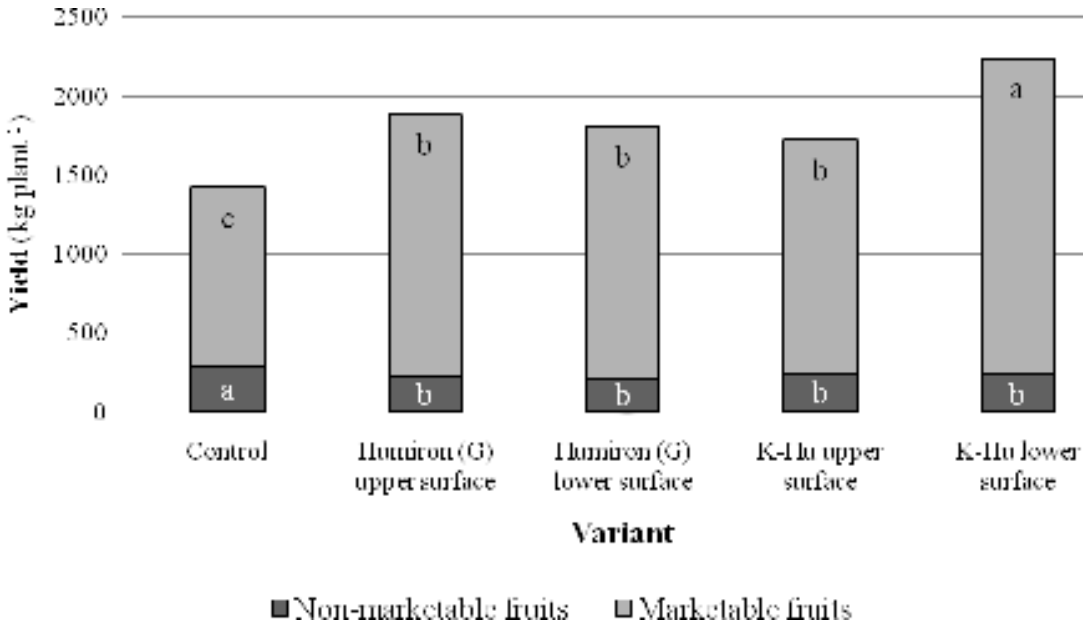


**Figure 5.17** Influence of different application forms of humates - upper and lower surface of the leaves - on yield of cucumber plants (four harvests). Tukey's HSD $p<0.05$. Different letters mean statistically significant difference.

As it was described in the literature review (page 24), application of humates contributes to a variety of different physiological reactions. Among those is increase in plants productivity which itself can be attributed to the establishment of the root system of the plants. An underlying factor here is the capability of the root system to uptake the nutrients. By creating conditions that support the development of the root system of the plant one can also influence the productivity of the cultivar.

**Discussion**

Foliar treatment can be beneficial in those cases when lack of micronutrients can inhibit development of plants. In this experiment with standard nutrient solution, there is no deficiency in any nutrient supply. The plants themselves can be susceptible to different kinds of diseases (mildew) and any application of humates can only deteriorate that situation. Particular in this experiment, general low productivity of plants and short vegetation period were due to outbreak of mildew. Thus, leaf treatments are only possible in combination with cultivars resistant to mildew. In all experiments where leaf treatments were undertaken, the problem of mildew arose. At the same time, treatments proved ineffective in stimulating productivity of plants. Humate treatments did brought comparable results of plants' growth and productivity but at the same time it fell short of expected standards that are common in industrial practices where

productivity of cucumber plants should not be less 15 kg plant$^{-1}$. It brings an assumption that leaf application of humates alone cannot be used in stabilizing functionality of plant's system.

### 5.2.2 Investigation of plant biostimulators in different applications

**Problem description**

In a previous experiment it was shown that the leaf application of biostimulators had a positive influence on the plants productivity, root length and plant growth. Use of different biostimulators as foliar fertilizers can lead to a rapid supply of plants with macro- and micronutrients in comparison to root treatments. Foliar application can increase intensity of metabolism within photosynthetic apparatus of the plants, which in turn results in higher yield of cucumber fruits. The scheme of the experiment is shown in the table 5.2.

**Table 5.2** Layout of the experiment

| Variant | Concentration | Application pattern | Quantity of solution |
|---|---|---|---|
| 1. **Control** | -* | - | Three times 20 ml plant$^{-1}$ |
| 2. **LACTOFOL** | 0.08% | Leaf application | |
| 3. **K-Humate** | 0.005% | Leaf application | |
| 4. *Bacillus subtilis FZB* 24® | Spore suspension (0.2%) | Leaf application | |
| 5. **LACTOFOL** | 0.08% | Watering in Substrate | |
| 6. **K-Humate** | 0.005% | Watering in Substrate | |
| 7. *Bacillus subtilis FZB* 24® | Spore suspension (0.2%) | Watering in Substrate | |
| 8. **LACTOFOL + K-Humate** + *Bacillus subtilis FZB* 24® (**Combined biostimulator**) | 0.08%+0.005%+0.2% (respectively) | Watering in Substrate | |
| 9. **LACTOFOL + K-Humate** + *Bacillus subtilis FZB* 24® (**Combined biostimulator**) | 0.08%+0.005%+0.2% (respectively) | Leaf application | |

*Variants without application

The functioning of the photosynthetic apparatus depends on the availability of water and minerals in the immediate microenvironment surrounding the root which can be delivered with nutrient solution and with additional biostimulator treatments. In this study we tested different application patterns (leaf, root) of bioactive substances (humate, lactate and *B.subtilis*) and their combinations on cucumber plants growth and productivity.

Application of biostimulating substances in the form of leaf treatment and root treatment had different effects on leaf quantity of development of cucumber plant cv. Indira (F1) (Figure 5.18).

Leaf quantity on the control variant was on average equal to 26. All leaf treatments with different biostimulating substances as well as their combination in form of Lactofol"O"+K-Humate+*B.subtilis* FZB 24 had inhibiting effect on the leaf formation in comparison to control variant. Root application with singular components of Lactofol "O", K-humate and *B.subtilis* FZB 24 showed on average 25, 28 and 27 leaves respectively. This result is not statistically significant in comparison with the control variant.

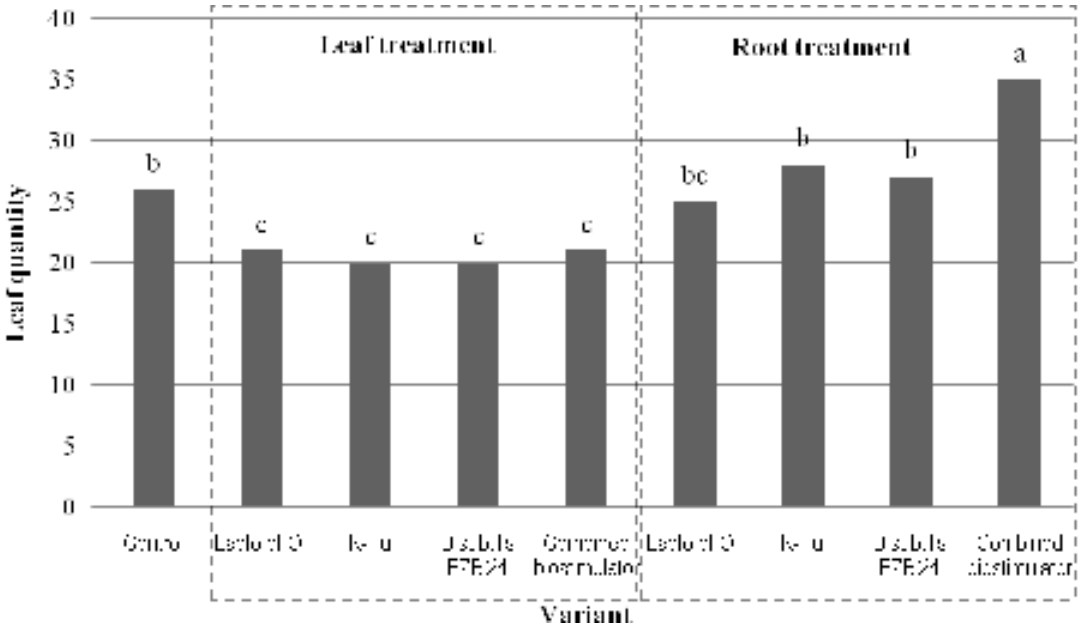


**Figure 5.18** Influence of different biostimulators and their application forms on leaf quantity of cucumber plants (four harvests). Tukey's HSD $p<0.05$. Different letters mean statistically significant difference. n=36

Use of a combined biostimulator showed the highest leaf count in the experiment – 35. Combined biostimulator showed very different results depending on application pattern. From the results of leaf quantity it can be deduced that primary use of Lactofol"O"+K-Humate+*B.subtilis* FZB 24 can be only in form of the root treatment.

Leaf area at the end of vegetation of cucumber plants is shown on the figure 5.19. Formation of leaf area of the cucumber plants was differently influenced depending on foliar or root treatment as well as formulation of biostimulating substances. The control variant recorded leaf area on the level of 3453 $cm^2$ $plant^{-1}$. Application of lactate, humate and *B.subtilis* and their combination, influence leaf area formation depending on their form and formulation, in both ways – increase and decrease. Use of Lactofol"O" as foliar fertilizer contributed to statistically significant increase of leaf area on this variant – 4322 $cm^2$ $plant^{-1}$ in comparison to control variant. Its

application as a root fertilizer showed insignificant change of the leaf area of cucumber plant on the level of 4567 $cm^2$ $plant^{-1}$.

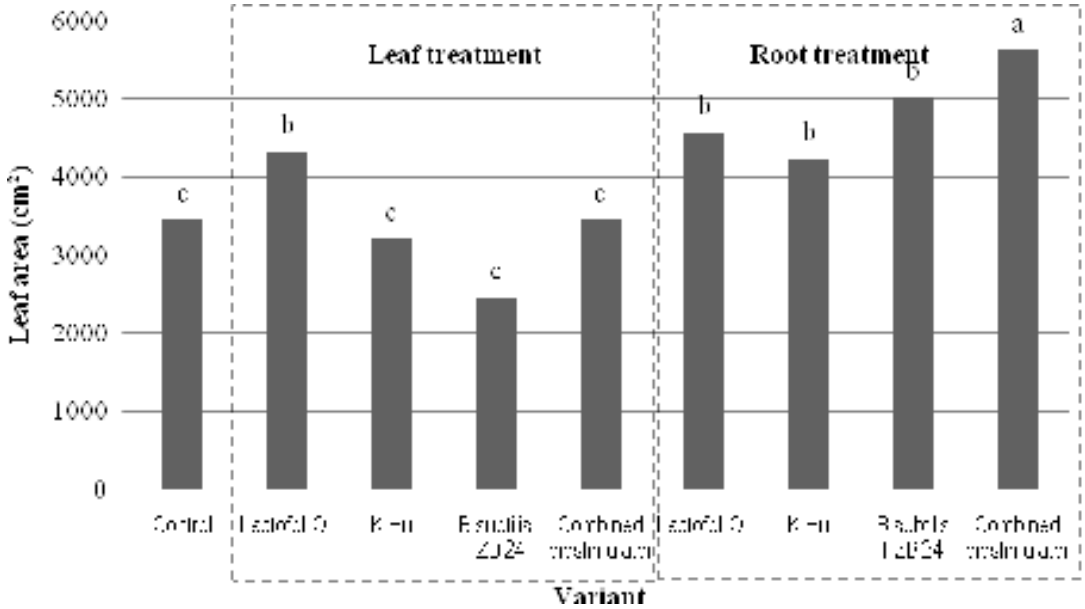


**Figure 5.19** Influence of different biostimulators and their application forms on leaf area of cucumber plants (four harvests). Tukey's HSD $p<0.05$. Different letters mean statistically significant difference. n= 36

K-Humate being applied on the leaves contributed to an inhibition of leaf area formation – 3211 $cm^2$ $plant^{-1}$ in comparison to its root application 4233 $cm^2$ $plant^{-1}$. *B.subtilis* in the variant with leaf treatment contributed to the lowest result in terms of leaf area development – 2456 $cm^2$ $plant^{-1}$. At the same time, application of *B.subtilis* FZB 24 to the root system of the cucumber plants played into increase of leaf area to the level of 5012 $cm^2$ $plant^{-1}$. This results is statistically significant once compared with both control and leaf treatment in the variant with *B.subtilis*.

The combined biostimulating mixture applied on the variant with root treatment created conditions that contributed to formation of the leaf area of plants. The plants on this variant showed 5631 $cm^2$ $plant^{-1}$ leaf area – the highest, statistically significant value in the experiment. The results of the experiment imply that application of *B.subtilis* as foliar fertilizer is not effective in an increase of the leaf area.

Fresh matter content in stems and leaves are shown in figure 5.20. Biomass synthesis of leaves and stems over vegetation time is a critical parameter for generation of functional assimilation apparatus of the plant (BERGMANN, 1992). Use of a combined biostimulator on the variants with root treatment showed the best result in comparison with other variants of the experiment.

The same combination of biostimulating substances applied on the leaves of the plants did not increase fresh matter content of stem biomass in comparison to control variant. Formation of leaf biomass was inhibited by application of combined biostimulator on the variants with foliar applications of the mixture. The other treatments, regardless of substance and application pattern showed the same results of the leaf area formation.

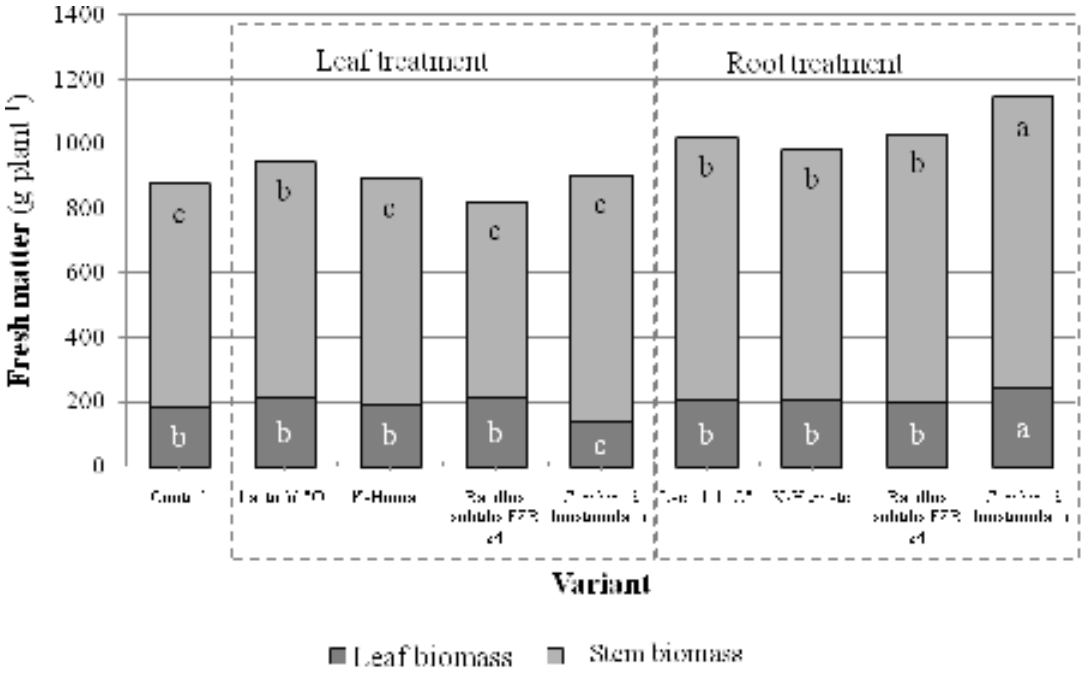


**Figure 5.20** Effects of biostimulator application (Lactate, K-Humate, *Bacillus subtilis FZB24*) on leaves and roots respectively on biomass of stems and leaves fresh matter. Different letters indicate significant differences (LSD, p=0,05). n= 36

Treatment of the plants with plant strengthening substances or biostimulators finds its effects in changed growth patterns. Bioactive substances differentiate development of plant tissues that results in increase in biomass of particular organs. Influence of different treatments has statistically significant effect on the variants with a combined biostimulator (K-Humate (0.01%) + LACTOFOL "O"(0.1%) + *B. subtilis FZB* 24® (0.2%)) (Figure 5.21). The same formulation of combined biostimulator applied on leaves did not produced any positive outcome in terms of fresh matter gain. Leaf biomass in this variant was lowest of all.

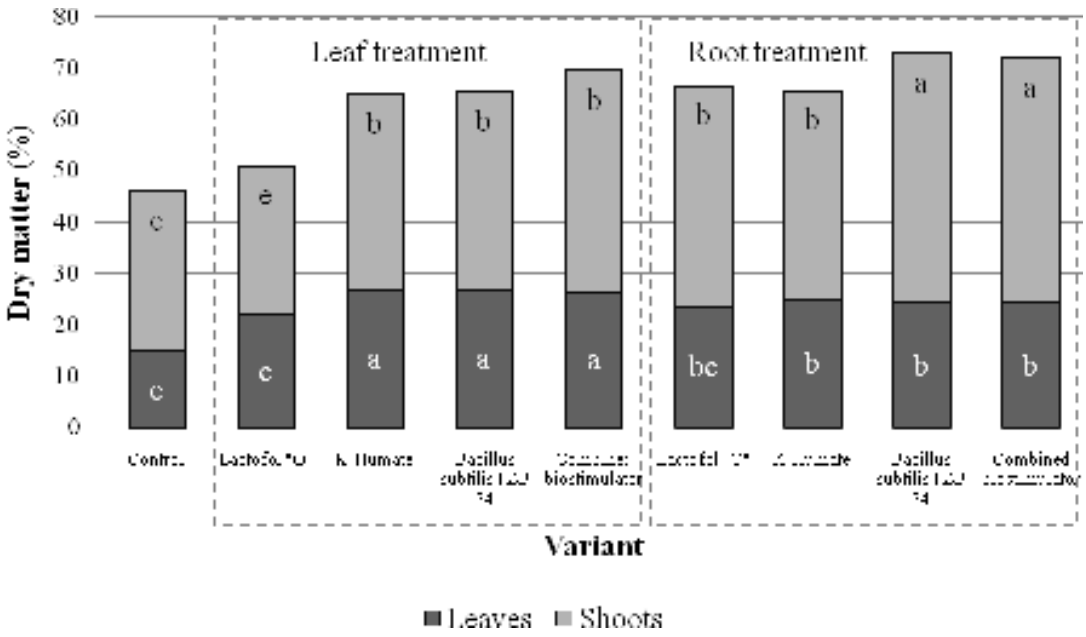


**Figure 5.21** Effects of biostimulator application (Lactate, K-Humate, *Bacillus subtilis FZB24*) on leaves and roots respectively on biomass of stems and leaves dry matter. Different letters indicate significant differences (LSD, P=0.05). n= 40

Leaf treatments by K-Humate, *B.subtilis* FZB 24, and combined biostimulator caused highest dry matter content in leaves and the same effect was achieved no the variant with root application, but in this case it was due to application of LACTOFOL"O" and K-Humate. The highest dry matter content in stems is observed on variants with root application of *B.subtilis* FZB 24 and combined biostimulator.

The number of fruits on the cucumber plants was higher on the variants with the root treatments (Figure 5.22). The highest fruit number was harvested on the variants with root application of *B.subtilis* and LACTOFOL "O" respectively. Although, leaf application of biostimulating substances can contribute to supply of nitrogen, iron and different micronutrients (ALEXANDER, 1986) it did not improve a productivity of the cucumber plants in the experiment.

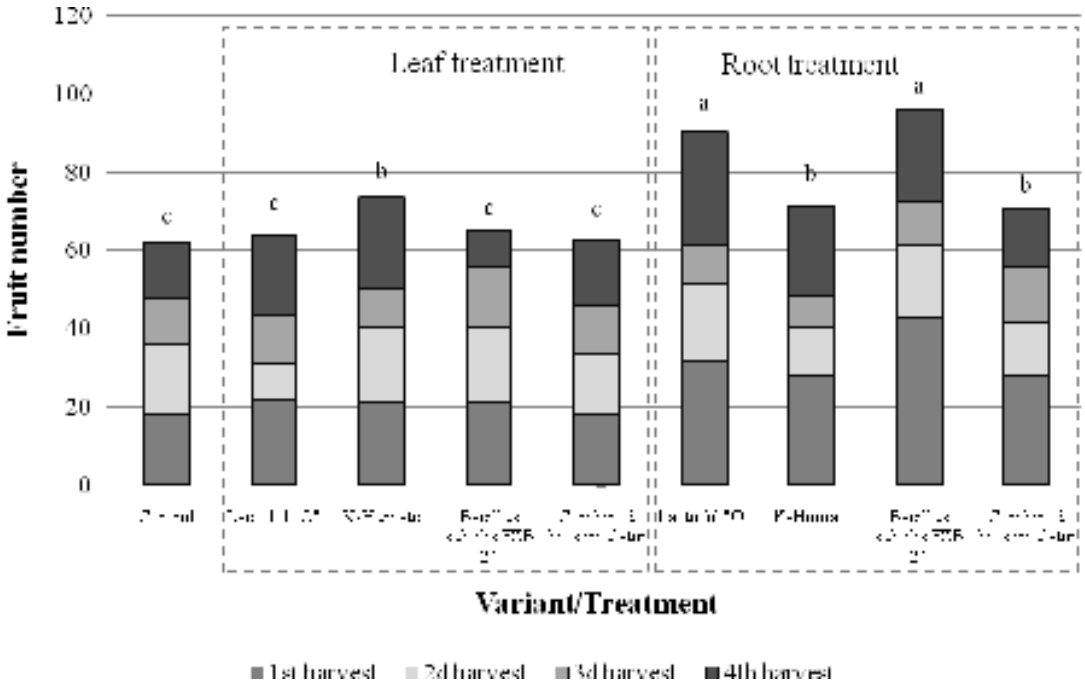


**Figure 5.22** Effect of application biostimulators (Lactate, K-Humate, *Bacillus subtilis*) on leaves and roots respectively on number of marketable fruits in four harvesting periods of 9 days each. Different letters indicate significant differences (LSD, P=0.05). n=27

The lowest percent of non-marketable fruits is seen on variants with root application of combined biostimulator (Figure 5.23). It was not statistically significant in comparison with the same variant of leaf treatment. Application of LACTOFOL "O" 0.01%, *B.subtilis* FZB24 0.2% and K-Humate 0.001% proved to be effective in increase of leaf and Stem biomass (fresh matter) only in case of root treatment. Leaf application did not have statistically significant differences between treatment with LACTOFOL "O", K-Humate and *B.subtilis*.

Effects induced by LACTOFOL "O" 0.01%, *B.subtilis* FZB24 0.2% and K-Humate 0.001% in different treatments resulted in different growth patterns of cucumber plants. Root application of biostimulating substances proved to be more effective in increasing dry matter content of leaves and stems and at the same time did not have any statistically significant difference on dry matter content of fruits.

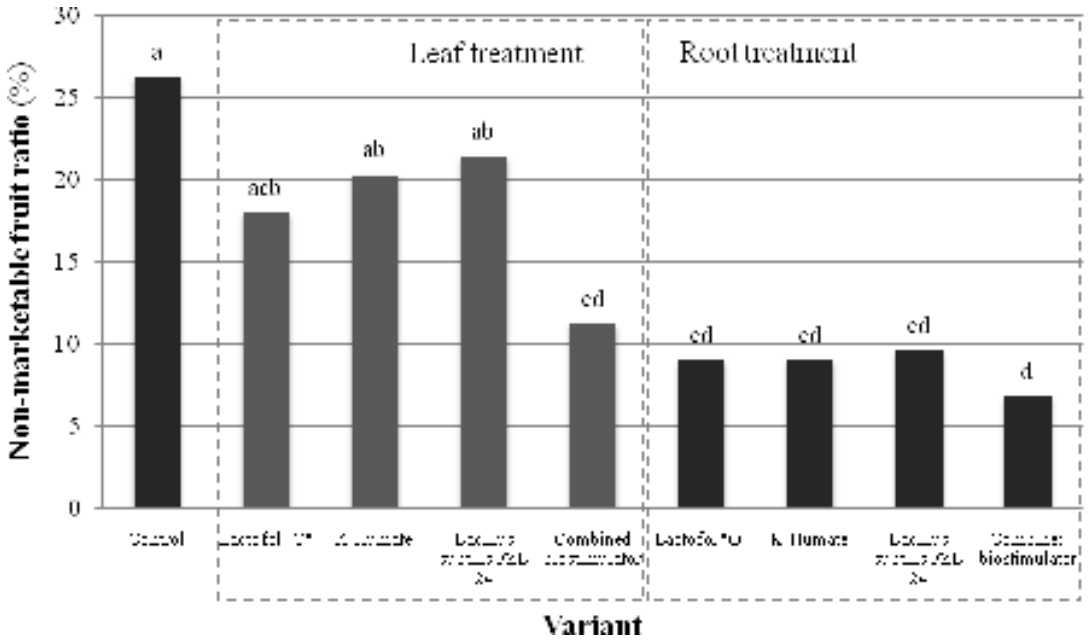


**Figure 5.23** Effect of application biostimulators (Lactate, K-Humate, Bacillus subtilis) on the percentage on non-marketable fruits. Different letters indicate significant differences (Chi-square-test, p=0.05). n=27

Effects induced by LACTOFOL "O" 0.01%, *B.subtilis* FZB24 0.2% and K-Humate 0.001% in different treatments resulted in different growth patterns of cucumber plants. Root application of biostimulating substances proved to be more effective in increasing dry matter content of leaves and stems and at the same time did not have any statistically significant difference on dry matter content of fruits. Application of combined biostimulating mixture significantly reduced number of non-marketable fruits. Variants with the root treatment showed significantly lower fraction of c-class cucumbers in comparison to variants with leaf treatments except the leaf treatment variant with combined biostimulating substance. Leaf treatments contributed to development of mildew on cucumber plants that resulted in very short vegetation time and low productivity. The application of all substances tested stimulated the stem development represented by a higher fresh matter of stems and leaves in most variants. Obviously the application pattern was important for the effect of the biostimulators. The application in the root zone led in each case to a higher fresh matter compared to the control. If the substances were applied over the leaves the effect on stem fresh matter was not as strong as if they were applied in the root zone. The application of *Bacillus subtilis* even resulted in a lower stem fresh matter. The fruit dry matter in both application forms, foliar and rhizospheric showed similar results (Figure 5.24).

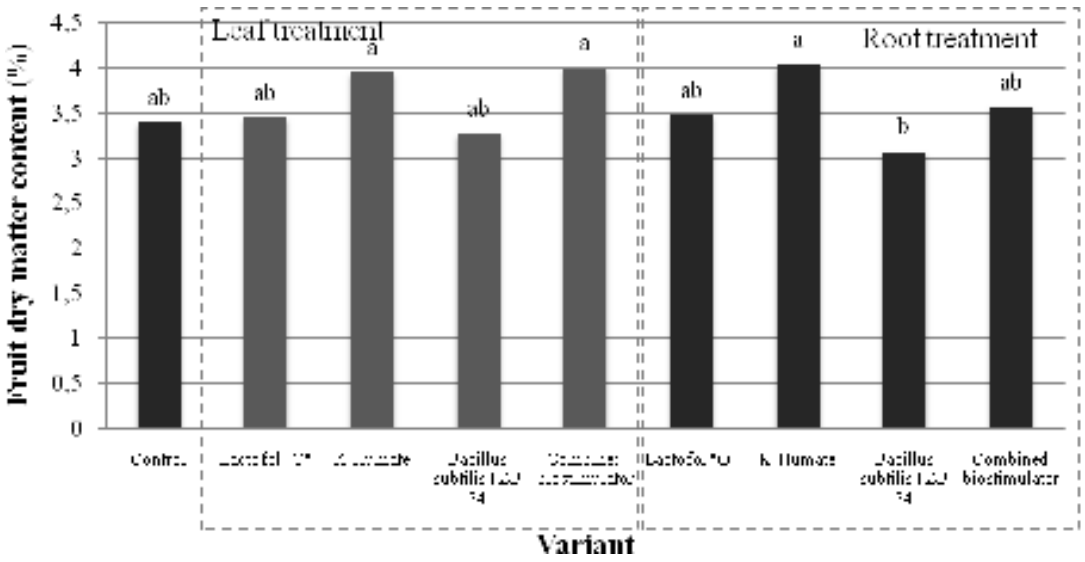


**Figure 5**.24 Effect of application biostimulators (Lactate, K-Humate, *Bacillus subtilis*) on leaves and roots respectively on dry matter content of marketable cucumbers. Different letters indicate significant differences (LSD, p=0.05). n=36

The effect on leaf fresh matter was also a stimulating one. It should be stressed, however, if the combination of all substances was applied the effect was opposite stimulating if applied over the roots and inhibiting if applied over the leaves.

**Discussion**

The application of biostimulators enhanced in most cases the dry matter content of stems and leaves. Therefore also the quality of stems and leaves seems to be different and effects on the weakness against fungi's could be expected. This effect was also found in experiments with water spinach (HOANG, 2003), however, in these experiments the effect on the root growth was much stronger than on the stem growth. In this respect much more investigations are necessary and the results are only a first advice. Comparing the ratio between stem and leaf fresh matter after application of biostimulators via roots more or less the same ratio was found as in the control indicating the stem and leaf growth was stimulated in the same manner. After application of biostimulators to the leaves the leaf growth was more encouraged than stem growth resulting in a lower ratio apart from leaf treatment with the combination of all substances. In this treatment leaf development was inhibited and therefore the stem/leaf ration increased.

## 5.3 Influence of combined biostimulating mixture on growth of cucumber plants

The growing medium used in container culture must have good nutrient- and water-holding characteristics, and provide good aeration to the root system. Fertilizer programs for soilless-culture systems must supply all nutrients required by the plants. At the same time, long term use of horticultural substrates creates specific conditions for plant's development. This conditions influence development of particular organs of plant (root, leaf formation, leaf area) as well as functioning of entire plant system.

### 5.3.1 Use of biostimulating mixture in hydroponical substrate culture

**Problem description**

The experiments with suboptimal growing factors demonstrated the potential of the biostimulating mixture to reduce influence of abiotic stress conditions on the cucumber plants. This experiment was designed to test hypothesis that application of biostimulating mixture can have different effects as a result of different properties of substrates. The long term use of horticultural substrates (perlite, rockwool, coir, peat, sheep wool) influences their physical and chemical properties. Change in specific properties of substrates inevitably influences development of horticultural crops. In practical terms, it finds its reflection in formation of yield of test plants. It is also very important to know the change pattern in properties of horticultural substrates. It means that initial properties of the substrates are not equal. They differ in such important characteristics like water holding capacity, cation exchange capacity, etc. Evolution of some of these characteristics has an important economic underpinning for commercial horticultural enterprise. Being able, under given conditions, to answer the question of substrate substitution time, can decide on commercial profitability of the greenhouse.

A combined biostimulating mixture with formulation Lactofol "O" 0.2% + K-Humate 0.1% + B.subtilis FZB 24 0.2% in quantity of 300 ml was applied to root systems of the test plants (*Cucumis sativus L*) cv. Indira (F1). It effected development of leaves of cucumber plant in for vegetations (Figure 5.3). All variants show an increase of leaf number of cucumber plants with every next vegetation period. Variant with perlite substrate showed no statistically significant differences between treated and non treated plants during first the vegetation. Formation of assimilation apparatus of the plants during the first vegetation on all variants with treated and non treated plants demonstrated lowest results in comparison with every next vegetation. Treated plants on the variant with perlite in the second vegetation period exhibit statistically significant increment in leaf number in comparison to non treated plants and to previous vegetation.

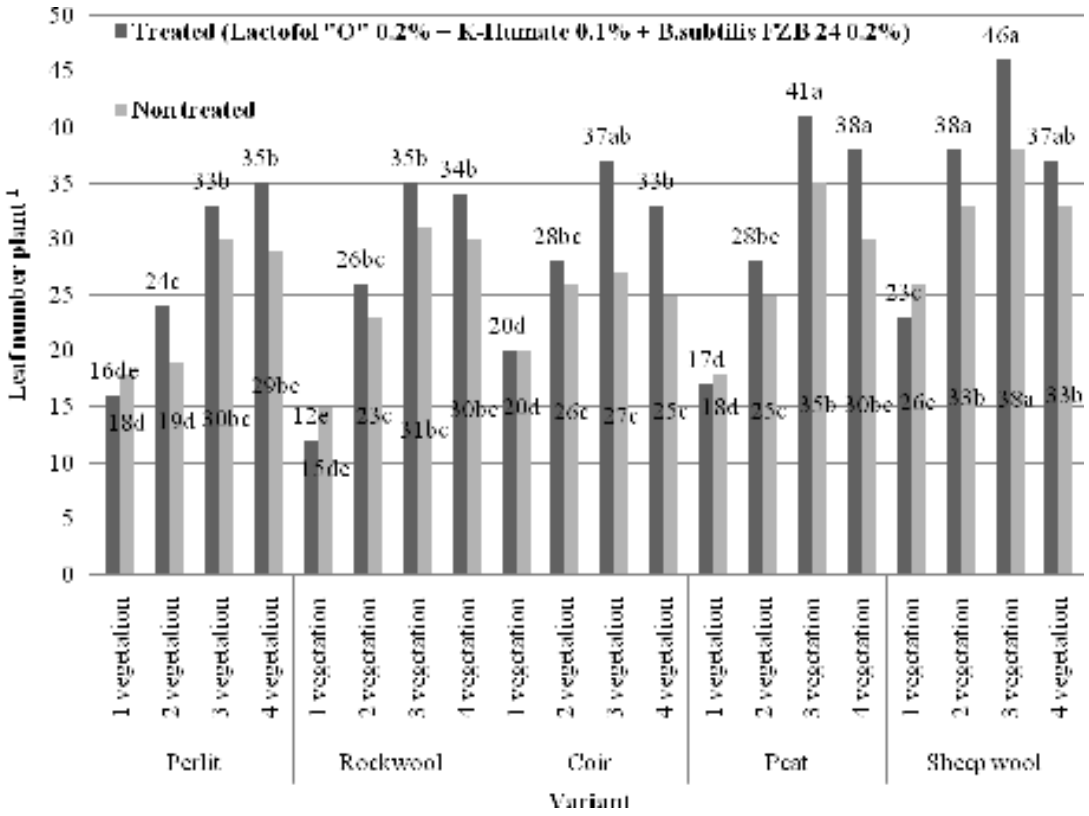


**Figure 5.30** Influence of combined biostimulating mixture on leaf number of cucumber plant. Two-way ANOVA (Tukey $p<0.05$). Different letters indicate significant differences.

The maximum leaf number on this variant was achieved in third and forth vegetations. These results coincide with observation on the variant with rockwool, coir during the same time period. The highest leaf count was recorded on the treated variants with coir substrate in third and forth vegetation and the variant with sheep wool.

At the same time, development of leaf area of cucumber plants can be divided into two periods – one with very small leaf area, and second with fully fledged leaf area (Figure 5.31). Data of leaf area at the end of every vegetation experiment represent the general situation in plant development. In the first vegetation period on all substrates and regardless of treatments plant exhibit leaf area between 2.32 $m^2$ $plant^{-1}$ – variant with perlite substrate without treatment and 2.7 $m^2$ $plant^{-1}$– variant with coir with treatment. An explanation for such drop in plants growth is the fact that rapid decrease of air temperature during the night substantially inhibited biological processes within the plant system.

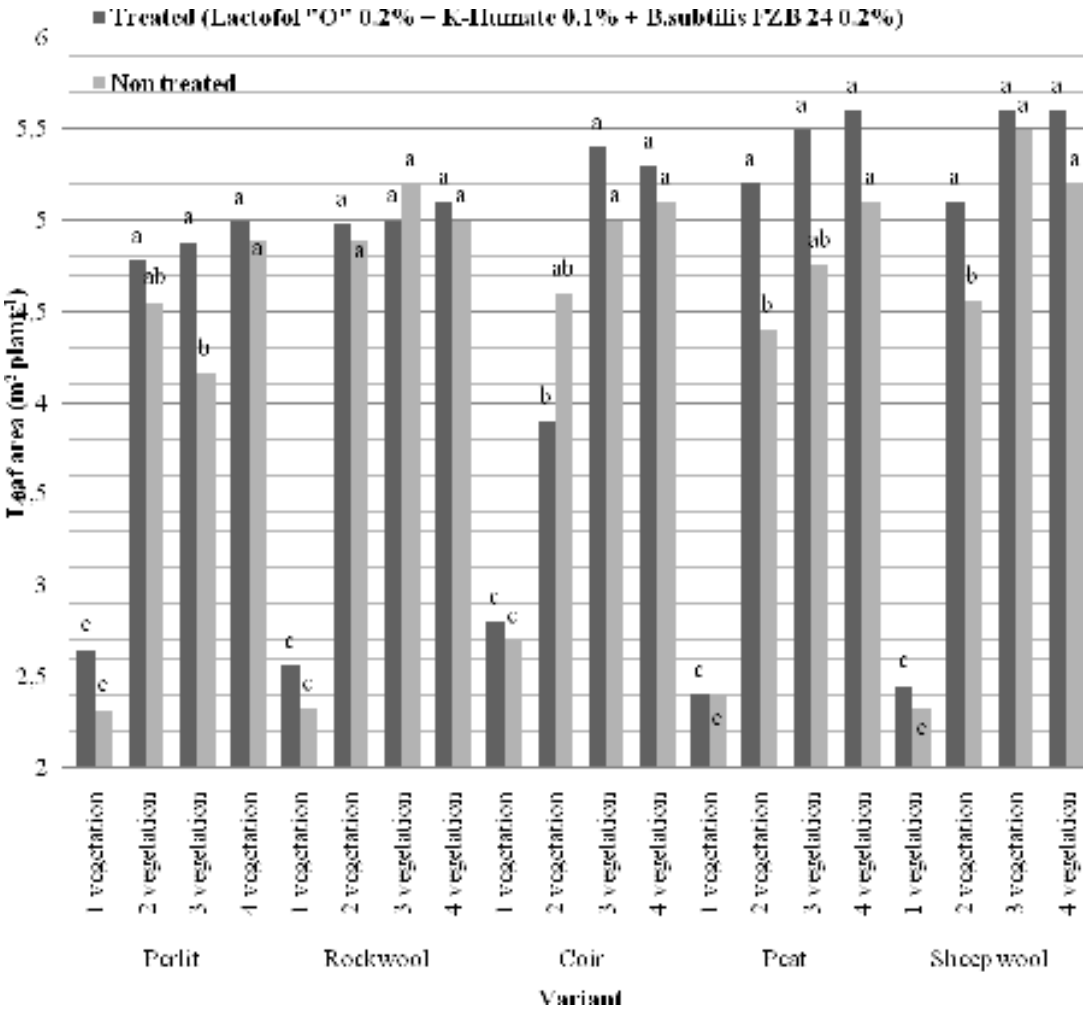


**Figure 5.31** Influence of combined biostimulating mixture on leaf area of cucumber plant. Two-way ANOVA (Tukey $p<0.05$). Different letters indicate significant differences.

The second vegetation showed statistically significant inference in increase of leaf area values in comparison to first vegetation on all variants. Results on variant with rockwool, coir, peat, sheep wool during the third and the fourth vegetation showed results that do not statistically differ. This statement is valid for treated and non treated plants. It means that application of biostimulating mixture did not influence leaf area formation at the end of vegetation. The substrate, as factor contributing to plants development, did not show significant influence between different types of horticultural substrates.

Figure 5.32 illustrates results of the plant length in the experiment. According to these data, there are statistically significant differences on the variants with treated plants. Plants grown on coir, peat and sheep wool were the highest in the experiment with treatment. Only on the variant with peat in forth vegetation a non-treated variant grown into the length that is statistically insignificant in comparison with treated plants.

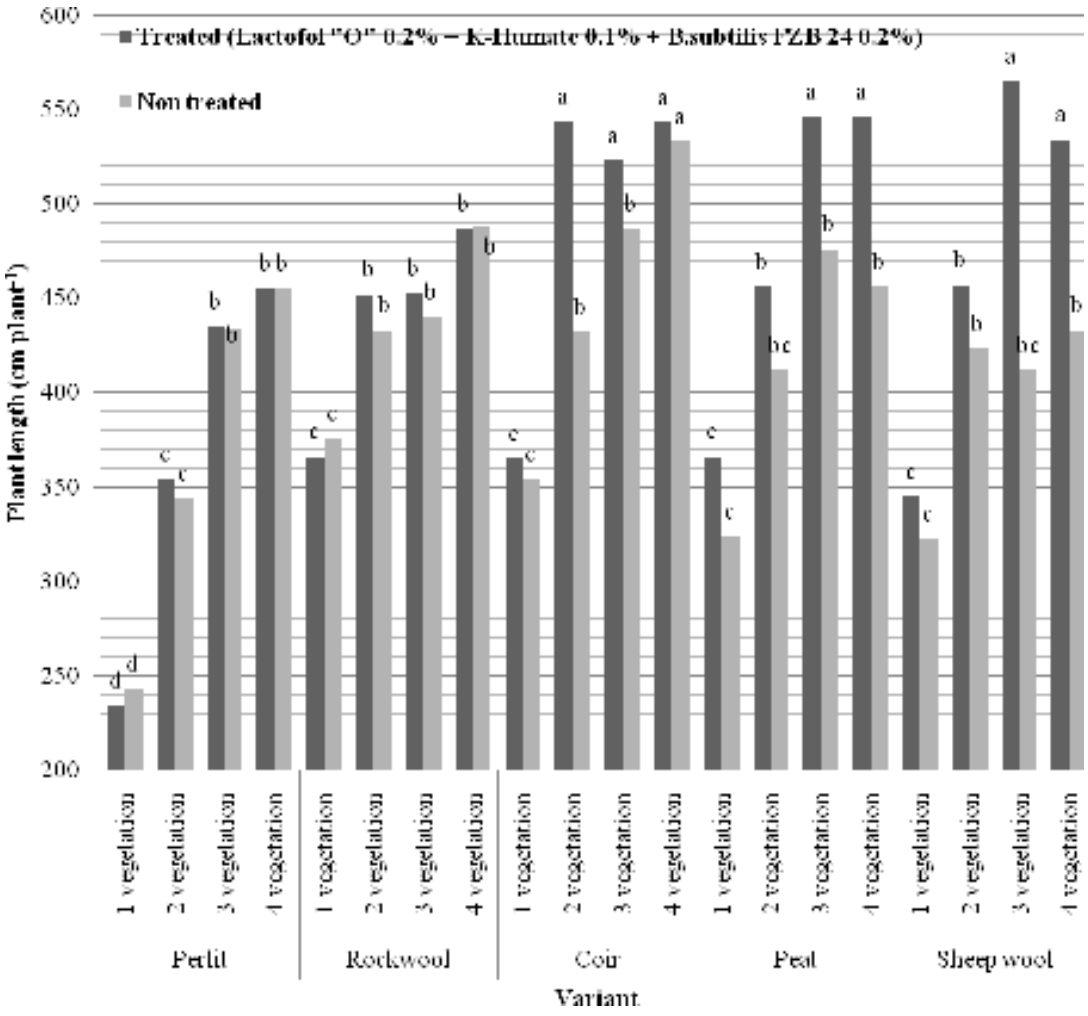


**Figure 5.32** Influence of combined biostimulating mixture on length of cucumber plant. Two-way ANOVA (Tukey $p<0.05$). Different letters indicate significant differences.

In general, application of biostimulating substances influenced the length of the test plants in this experiment. The development of plants was influenced by both – substrate type and treatment, but at the same time, factorial analysis show that plants cultivated on coir, peat and sheep wool was influenced by the treatment more than by the type of substrate itself. This statement is valid for the second, third and fourth vegetation period in the experiment. Harvested cucumber fruit yield data resides in the tables (5.30 and 5.31). Statistical comparison of the yield data show that highest yield was recorded on the variants with peat and coir. The yield of the cucumber fruits on different variants was influenced by two factors: type of the horticultural substrate and treatment with biostimulating mixture consisting of K-Humate 0.01%+LACTOFOL "O" 0.1%+ *B.subtilis* FZB24 0.2%.

The variants without treatment produced the highest yield in the fourth vegetation except the variant with the sheep wool as a horticultural substrate. On this variant, the highest yield was observed during the third vegetation

**Table 5.30** Cucumber yield on sheep wools compared with coir, rockwool and perlite and compared un-treated and treated. Different letters indicate significant differences (Tuckey $_{0.05}$; comparison between variants)

| **Substrates** | **Yield** (kg plant$^{-1}$) | | | |
|---|---|---|---|---|
| | **First vegetation** | | **Second vegetation** | |
| | **untreated** | **treated** | **untreated** | **treated** |
| **Sheep wool** | 1.08 ± 0.35 bcd | 1.94 ± 0.43 a | 8.96 ± 0.72 bc | 10.07 ± 0.19 a |
| **Peat slabs** | 0.57 ± 0.17 a | 1.28 ± 0.55 bc | 7.49 ± 0.13 c | 10.41 ± 0.10 a |
| **Coir** | 0.76 ± 0.19 bc | 0.84 ± 0.27 bc | 7.51 ± 0.26 c | 8.09 ± 0.08 b |
| **Perlite** | 0.77 ± 0.33 d | 1.16 ± 0.61 bc | 6.68 ± 0.04 d | 8.95 ± 0.20 b |
| **Rockwool** | 1.47 ± 0.24 bc | 1.71 ± 0.06 cd | 6.11 ± 0.32 d | 9.16 ± 0.18 b |

*Untreated* – no application of biostimulators; *Treated* – application of biostimulators 0.01% K-Humate, 0.2% LACTOFOL"O", spore suspension 0.2% ($10^7$ cfu ml$^{-1}$) of *Bacillus subtilis* FZB 24®

The variants of the experiment without application of the biostimulating mixture produced lower yields in comparison to treated variants. Productivity of the cucumber plants over four vegetation periods was increasing The last fourth vegetation with additional treatment of the plants with the biostimulating mixture (table 5.31) showed the highest yield of the cucumber fruits with comparison to any other growing period of this experiment, except the variant with sheep wool. On the variant with the sheep wool substrate and application of the biostimulating mixture the highest yield was recorded during the third vegetation (11.27±2.11 kg plant$^{-1}$). In the fourth vegetation, there was no statistically significant difference between treated and not treated variants on the peat and perlite substrates.

**Table 5.31** Cucumber yield on the sheep wool compared with coir, rockwool and perlite and compared un-treated and treated. Different letters indicate significant differences (Tuckey $_{0.05}$; comparison between variants)

| **Substrates** | **Yield** (kg plant$^{-1}$) | | | |
|---|---|---|---|---|
| | **Third vegetation** | | **Forth vegetation** | |
| | **untreated** | **treated** | **untreated** | **treated** |
| **Sheep wool** | 11.27±2.11 b | 13.70±0.33 b | 10.11±1.87 b | 11.52±1.01 d |
| **Peat** | 13.54±1.45 b | 16.70±1.12 a | 15.32±0.47 a | 17.32±0.87 a |
| **Coir** | 12.72±1.44 b | 15.50±1.17 a | 14.50±1.07 b | 16.11±0.32 a |
| **Perlite** | 8.33±1.71 b | 13.40±1.56 b | 13.92±2.22 ab | 14.34±0.89 ab |
| **Rockwool** | 9.61±0.44 b | 10.23±1.22 c | 10.12±0.33 b | 13.31±1.22 c |

*Untreated* – no application of biostimulators; *Treated* – application of biostimulators 0.01% K-Humate, 0.2% LACTOFOL"O", spore suspension 0.02% ($10^7$ cfu ml$^{-1}$) of *Bacillus subtilis* FZB 24®

The comparison of the yield between variants shows that treatments with the mixture of biostimulating substances can play a decisive role after occurrence of the suboptimal growing conditions. In this particular case, the suboptimal growing factor was air temperature during the night.

Results of this experiment analyze the influence of combined biostimulating mixture (K-Humate 0.01%+LACTOFOL "O" 0.1%+ *B.subtilis* FZB24 0.2%) on properties of horticultural substrates as perlite, rockwool, coir, peat and sheep wool in the long term use (four fruit rotations). The changes and comparison of the physical properties such as air capacity (AC), water capacity (WC) and pore volume (PV) on the treated variants of the experiment are shown in the table 5.32-5.33.

Development of the AC during the vegetation on the treated variants shows that at the beginning of the vegetation, the highest value was obtained on the variant with sheep wool. This value was stable during the four vegetations and was at the level of 69-78%. The lowest air capacity was registered in the peat – 18% at the start of the vegetation. This value rose during the use of the substrate to 37%. Coir showed its lowest air capacity after second vegetation – 20% and its maximum was achieved after fourth use – 35%. Perlite had 58% of air capacity before its use as a substrate in the experiment. The absolute minimum was achieved after the second vegetation – 41%. Rockwool substrate had its lowest air capacity after the second vegetation – 17%, which was the lowest in the experiment.

**Table 5.32** Physical properties of substrates. Different letters indicate significant differences (Tukey $p<0.05$) within one parameter. Treated variants.

| Substrate | Before use | | | After second use | | |
|---|---|---|---|---|---|---|
| | AC (%) | WC (%) | PV (%) | AC (%) | WC (%) | PV (%) |
| **Sheep wool** | 69.40±1.51f | 22.80±1.72a | 96.80±1.30e | 43.10±3.18cd | 44.10±3.53cd | 87.20±2.99abc |
| **Peat** | 18.00±1.82a | 68.00±3.55fg | 86.00±2.82ab | 30.70±4.44b | 61.60±3.83f | 92.30±2.00d |
| **Coir** | 30.60±2.37b | 52.80±2.27e | 83.90±3.42a | 20.00±4.94a | 72.30±4.47g | 92.30±3.4d |
| **Perlite** | 58.60±1.51e | 31.60±1.51b | 90.20±0.82bcd | 41.40±3.50c | 50.40±3.40e | 91.80±1.81d |
| **Rockwool** | 49.20±9.98 d | 41.60±10.25c | 90.70±0.83cd | 17.20±4.32a | 74.60±3.80g | 90.10±2.42bcd |

AC – air capacity, WC – water capacity, PV - pore volume

Changes in WC of the substrates over the four different vegetation periods are connected to the origin and the nature of the horticultural substrates. The organic and inorganic substrates are exposed to the biological activity of the test plants, microbial community and mechanical stresses such as the regular wetting by the nutrient solution. The water capacity of the sheep wool before the use in the experiment on the treated variants was 22.80%. After the first vegetation this value nearly doubled and was at the level of 44.10%, which is the maximum value during the entire experiment (table 5.32). The values of the water capacity after the third and fourth vegetation were 25.30 and 23.20% respectively. The peat substrate had the highest water holding capacity of all the substrates used in the experiment, except coir. After the second vegetation WC of the peat and coir were 92.30%. On the variant with perlite with the treatment by the biostimulating mixture the minimum value of WC was obtained before the use, and its maximum value was after the third use – 50.50%. The same substrate showed the minimum value of WC before being used in the experiment – 31.60%. The variant with the rockwool within the treated half of the experiment improved its water holding capacity from 41.60% before the use to 77% at the end of the fourth vegetation.

Transformation of the pore volume (PV) on the treated variants was observed during the four vegetations of the cucumber plants on the variants with application of the biostimulating mixture and without it. The highest pore volume recorded before the use of the substrates was registered on the sheep wool (96.80%) on the variants with treatment (table 5.32). The value of the pore volume on the substrate was lower after the second vegetation 87.20%. After the third vegetation, the porosity of the sheep wool decreased to 80.20% (table 5.33). The results obtained after the fourth rotation, showed that the value of the pore volume on the sheep wool increased (88.20%). The lowest pore volume among the substrates used in the experiments was registered

by coir (83.90%). This value however, manifested an upward trend during the second and third vegetations 92.30 and 92.70% respectively. The fourth use of the substrate led to the decrease of the porosity to 60.10% (table 5.33).

**Table 5.33** Physical properties of substrates. Different letters indicate significant differences (Tukey $p<0.05$) within one parameter. Treated variants.

| Substrate | After third use | | | After forth use | | |
|---|---|---|---|---|---|---|
| | AC (%) | WC (%) | PV (%) | AC (%) | WC (%) | PV (%) |
| **Sheep wool** | 77.20±4.31 a | 25.30±1.12 d | 80.20±1.67 b | 78.30±1.23 a | 23.20±2.23d | 88.20±2.23 a |
| **Peat** | 35.30±2.12 c | 75.20±1.56 a | 96.60±3.12 a | 37.40±2.13 b | 72.00±2.65 a | 57.30±5.23 b |
| **Coir** | 30.70±3.56 c | 68.30±4.23 b | 92.70±2.23 a | 35.10±2.71 b | 68.30±1.76 b | 60.10±1.33 b |
| **Perlite** | 45.20±3.12 b | 50.50±2.76 c | 95.10±5.11 a | 55.20±3.12 c | 45.20±3.22 c | 88.30±3.2 a |
| **Rockwool** | 18.60±1.11d | 76.20±4.12 a | 70.50±3.81 c | 22.10±1.11 d | 77.30±5.11 a | 67.20±1.15 d |

AC – air capacity, WC – water capacity, PV - pore volume

Development of the physical properties of the substrates on the variants without treatment is described in the tables 5.34 and 5.35. The physical properties – air capacity, water capacity and pore volume in comparison between new substrates (before use) and after first vegetation on the variants without treatment show statistically significant differences between some of the variants.

**Table 5.34** Physical properties of substrates. Different letters indicate significant differences (Tukey $p<0.05$) within one parameter. Variants without treatment.

| Substrate | Before use | | | After first use | | |
|---|---|---|---|---|---|---|
| | AC (%) | WC (%) | PV (%) | AC (%) | WC (%) | PV (%) |
| **Sheep wool** | 65.80±2.22 a | 40.20±3.56 d | 86.30±0.45d | 65.30±2.32 a | 37.70±3.21d | 70.30±3.83c |
| **Peat** | 19.30±1.41f | 85.20±3.21 a | 90.40±0.56b | 25.30±0.22d | 77.30±1.87a | 83.00±2.77a |
| **Coir** | 35.60±2.13d | 60.30±1.27 c | 88.30±1.23c | 40.10±1.67c | 66.40±2.11b | 84.10±1.33a |
| **Perlite** | 56.20±1.15 b | 34.30±1.34 e | 94.10±2.31a | 59.70±1.11b | 38.10±1.17d | 77.80±3.12b |
| **Rockwool** | 22.70±3.11c | 80.20±4.42 b | 92.20±0.73a | 33.20±4.12b | 71.20±3.21c | 83.10±2.13a |

AC – air capacity, WC – water capacity, PV - pore volume

The physical properties of sheep wool differ from the other organic substrates especially before use. The air capacity exceed the recommended target values of 30 to 40% AC while the water capacity is much lower than the target values of 45 till 50 % WC. The low water capacity in the fresh substrate could be due to the high amount of fats in the unclean wool. The increase of water capacity during the cultivation period indicates that by the application of the acid nutrient solution for some month the fat can be washed out and the water capacity can increase. The low water capacity of sheep wool provides no buffering of the substrates for periods with low water availability.

**Table 5.35** Physical properties of substrates. Different letters indicate significant differences (Tukey $p<0.05$) within one parameter. Variants without treatment.

| Substrate | After third use | | | After forth use | | |
|---|---|---|---|---|---|---|
| | AC (%) | WC (%) | PV (%) | AC (%) | WC (%) | PV (%) |
| **Sheep wool** | 57.60±1.36a | 42.00±2.16 c | 68.30±2.13 c | 55,20±1,34a | 42.30±0,67 c | 67.10±0.12 c |
| **Peat** | 36.70±3.67 c | 68.70±2.34 b | 69.00±1.23 c | 39,30±2,23c | 65.30±1.11a | 62.30±1.45 b |
| **Coir** | 34.20±2.67 c | 70.30±1.23 a | 70.20±2.34 b | 38,10±1,13c | 62.30±3.22 b | 72.30±3.21a |
| **Perlite** | 41.30±2.34 b | 66.30±2.33 b | 70.20±1.31 b | 42,30±2,13b | 58.10±1.88 b | 65.60±2.38 b |
| **Rockwool** | 35.60±1.11d | 70.30±1.77a | 75.50±1.87 a | 37.10±1.23c | 65.10±2.21a | 63.30±2.87 b |

AC – air capacity, WC – water capacity, PV – pore volume

The pore volume of sheep wool was significantly higher than that of the other substrates. After the second use of the substrates the pore volume decreased for sheep wool. For peat and coir that means for the other organic substrates the pore volume increased while for inorganic substrates there was no change in the pore volume after use. The air capacity of sheep wool decreased after use resulting in a higher water capacity. Peat slabs had a low air capacity. This low air capacity of peat slabs before planting can be due to the high density of this material, because for easy transporting the slabs are dried and pressed. After wetting, the water capacity was saturated very fast. The air capacity, however, increased slowly over the vegetation period. Coir with very long fibers had a mean air capacity. The water capacity was also in the desired range. The air capacity decreased after the cultivation of cucumbers, while the water capacity increased. For perlite and rockwool slabs the expected values for air and water capacity could be measured before use. The decrease in air capacity of these substrates was as expected. The results from the table 5.36 testify about significant changes in the bulk density of the substrates during experiment's

lifetime. Comparing values at the beginning of the experiment and at the end of it, the conclusion is that after four plant rotations the compaction of the substrates increased.

**Table 5.36** Bulk density of the substrates (g*m$^{-3}$). Different letters indicate significant differences (Tukey p<0.05) within one parameter. Treated variants.

| Substrate | Before use | After first use | After second use | After third use | After forth use |
|---|---|---|---|---|---|
| **Sheep wool** | 78,0±0,81 b | 77,6±4,55 a | 71,0±0,81 a | 75,3±0,55 a | 78,1±3,43d |
| **Peat** | 133,6±5,31 c | 171,5±13,91 b | 177,6±2,25 c | 177,8±6,12 b | 180,2± 11,17 a |
| **Coir** | 124,9±2,34 c | 168,9±5,72 b | 176,1±5,42 c | 183,5±3,12 b | 185,5±12,22 a |
| **Perlite** | 132,1±4,65 c | 151,7±4,23 b | 106,6±2,56 b | 121,3±12,2 c | 140,3±3,12 b |
| **Rockwool** | 50,4±6,38 a | 70,0±12,24 a | 106,0±5,47 b | 100,5±3,31 d | 115,5±5,15 c |

The reason for the increase of the bulk density of the substrates can be attributed to the growth of the plants. Every plant rotation after being removed at the end of its biological vegetation period, the root systems of the plants remained in the substrates. The same situation can be observed on the variants without treatment with biostimulating substance (table 5.37). Utilization of perlite, rockwool, peat, coir and sheep wool as horticultural substrates during the four fruit rotations conferred its effect on their physical properties. Regardless of treatment (Table 5.36 and 5.37) all substrates show increase of bulk density – naturally occurring process due to the fact that every fruit rotation leaves its roots and foliage in the substrates. Regarding the parameter of the bulk density before the use, the substrates (Table 5.36 and 5.37) can be divided into two significantly different groups. The first group with sheep wool and rockwool has a low and second with all other substrates has a high bulk density. After the first use the bulk density of rockwool increased but did not exceed that of sheep wool. There was also a significant rise in the bulk density of the second group after the first use. Therefore the ratio between the two groups is the same. After the second use the bulk density of peat and coconut did not change, while the density of rockwool increased considerably. The increasing density in some of the substrates can be due to the high amount of roots accumulating in the root zone. The accumulation of roots could be also observed in sheep wool; however, there was no change in the bulk density. The structure of sheep wool was completely mixed with roots after the second cultivation and the root systems could be not clearly distinguished from the substrate. An extension cultivation period on sheep wool seems to be not possible.

**Table 5.37** Bulk density of the substrates (g*m$^{-3}$). Different letters indicate significant differences (Tukey p<0.05) within one parameter. Variants without treatment.

| Substrate | Before use | After first use | After second use | After third use | After forth use |
|---|---|---|---|---|---|
| **Sheep wool** | 80,2±3,87 c | 82,3±2,23 c | 75,5±2,34 d | 76,5±0,56 e | 80,3±4,23e |
| **Peat** | 137,3±2,66 a | 158,3±3,36 b | 163,6±2,19 c | 179,3±1,15 b | 183,2±1,89 a |
| **Coir** | 130,0±3,67 b | 167,6±2,45 a | 170,2±1,34 a | 183,3±1,13 a | 183,1±0,45 a |
| **Perlite** | 133,5±6,18 ab | 160,2±4,12 ab | 166,2±1,11 b | 130,1±1,76 c | 145,2±1,78 b |
| **Rockwool** | 55,3±3,12 d | 77,3±2,12 d | 79,3±1,12 d | 95,3±2,13 d | 95,78±1,67 c |

The analyses of the mineral content of the substrates (Table 5.38 and 5.39) showed that there was no accumulation of the nutrients in the sheep wool, therefore, indicating that there was no ion exchange capacity in the substrate. However, the ion exchange capacity has to be determined in following experiments. The small amount of coconut fibers included in the sheep wool substrate obviously did not affect the nutrient accumulation. In comparison with sheep wool in the nutrients in peat and coir were accumulated to a remarkable amount, especially potassium after the first vegetation and $NO_3$ and $Ca_2O$ after the second vegetation. Accumulation of N-$NO_3^-$ continued in third and forth vegetations.

Interaction of the substrates with nutrient solution, mainly its physical interaction like flooding of the substrates' container with nutrient solution and draining of the solute back to the tanks, contribute to the process of compaction of the substrate material and thus increase in the bulk density of the substrates. The substrates ability to retain some nutrients within its structure also affects the results of the nutrients content (table 5.38). The treated variants of the experiment showed different results in ability to retain nutrients owing to their physical properties combined with application of additional portion of the nutrients with lactate. Sheep wool retained the most of the nitrogen and calcium at the end of the fourth vegetation at the level of 38.3±1.34 and 34.6±1.77 ppm respectively. The maximum of the potassium concentration in the substrate was observed during the very first vegetation (28.4 ± 3.16 ppm). The concentrations of the nutrients on the peat substrate were 255.00±2.67 ppm of N-$NO_3^-$ in the last vegetation, 115.70 ± 3.27 ppm of potassium during the first vegetation and accumulation of the calcium was the largest in the fourth vegetation at the level of 80.50 ± 3.73 ppm.

The variant with coir as a substrate showed the highest concentration of nitrates in the third vegetation with 231.20 ± 7.71 ppm of N_$NO_3^-$. Maximum concentrations of potassium and calcium were observed at 113.20 ± 6.83 ppm in the first vegetation and 83.30 ± 1.23 ppm during

the last vegetation respectively. Perlite retained the highest amount of nitrates during the third vegetation 67.70 ± 1.45 ppm. Maximum concentrations on potassium and calcium were obsewrved ith second vegetation at the level of 56.60 ± 0.82 and 92.30 ± 6.18 ppm respectively.

**Table 5.38** Content of nutrients in the substrates of treated variants (ppm). Different letters indicate significant differences (Tukey $p<0.05$) within one parameter. Comparison between substrates.

| Substrate | Nutrients | First vegetation (ppm) | Second vegetation (ppm) | Third vegetation (ppm) | Forth vegetation (ppm) |
|---|---|---|---|---|---|
| **Sheep wool** | | 21.60 ± 3.12 a | 23.60 ± 1.34 a | 24.40 ± 1.21 d | 38.30 ± 1.34 c |
| **Peat** | | 77.90 ± 5.60 c | 77.10 ± 5.53 bc | 190,60 ± 3,12 b | 255.00 ± 2.67 a |
| **Coir** | **N-$NO_3^-$** | 68.20 ± 6.51 c | 85.20 ± 1.00 c | 231.20 ± 7.71 a | 170.00 ± 2.44 b |
| **Perlite** | | 34.70 ± 4.58 b | 65.60 ± 16.74 b | 67.70 ± 1.45 c | 38.40 ± 1.06 c |
| **Rockwool** | | 345.10 ± 4.43 d | 248.10 ± 5.55 d | 210.60 ± 8.78 a | 37.70 ± 1.11 c |
| **Sheep wool** | | 28.40 ± 3.16 a | 24.20 ± 1.00 a | 25.50 ± 1.71e | 15.20 ± 0.34 d |
| **Peat** | | 115.70 ± 3.27 d | 72.40 ± 8.76 c | 75.30 ± 0.41 c | 77.20 ± 3.45 b |
| **Coir** | **K** | 113.20 ± 6.83 d | 75.80 ± 2.06 c | 78.10 ± 1.89 b | 79,30 ± 1,56 b |
| **Perlite** | | 55.30 ± 0.59 b | 56.60 ± 0.82 b | 55.30 ± 0.12 d | 54.70 ± 2.45 c |
| **Rockwool** | | 70.40 ± 0.32 c | 235.90 ± 5.11 d | 156.80 ± 5.14 a | 112.50 ± 4.12 a |
| **Sheep wool** | | 33.80 ±1.55 b | 32.70 ± 2.00 a | 33.40 ± 0.23 e | 34.60 ± 1.77 d |
| **Peat** | | 64.90 ± 1.22 d | 73.70 ± 1.17 b | 70.20 ± 2.12 c | 80.50 ± 3.73 b |
| **Coir** | **Ca** | 55.70 ± 1.49 c | 82.90 ± 10.99 bc | 81.60 ± 3,32 b | 83.30 ± 1.23 b |
| **Perlite** | | 24.60 ± 1.30 a | 92.30 ± 6.18 cd | 55.60 ± 2.23 d | 56.20 ± 1.71 c |
| **Rockwool** | | 115.30 ± 3.00 e | 125.10 ± 3.56 b | 112.40 ±1.32 a | 125.7 ± 4.21 a |

The analysis of nutrients in the rockwool, during the four different vegetations, on the variants with application of the biostimulating mixture showed that the concentration of N-$NO_3^-$ was at its maximum during the first vegetation - 345.10 ± 4.43ppm. The highest concentrations of potassium and calcium were found during the second vegetation - 235.90 ± 5.11 and 125.10 ± 3.56 ppm respectively.

Development of root system of the plants inevitably leads to compaction of substrate material – the result of higher bulk density. Treated variants indicate accumulation of nutrient elements over vegetation time (Table 5.39). Not all substrates exhibit the same pattern. Sheep wool and peat show reduction in potassium content – the fact that can be attributed to physical properties of these substrates. All substrates except rockwool and perlite showed stable accumulation patter

of nutrients. This pattern can be explained by the different physical properties of inorganic materials. Specifically perlite and rockwool do not posses that retention capacity of peat or coir. Long term use of horticultural substrates (perlite, rockwool, coir, peat, and sheep wool) finds its manifestation in their physical properties.

**Table 5.39** Content of nutrient in the substrates of variants without treatment (ppm). Different letters indicate significant differences (Tukey $p<0.05$) within one parameter. Comparison between substrates.

| Substrate | Nutrients | First vegetation (ppm) | Second vegetation (ppm) | Third vegetation (ppm) | Forth vegetation (ppm) |
|---|---|---|---|---|---|
| **Sheep wool** | **$N\text{-}NO_3^-$** | 21.60 ± 3.12 a | 22.10±0.34 e | 36.30±1.23d | 35.20±2.11 c |
| **Peat** | | 77.90 ± 5.60 c | 100.00±2.23 b | 133.50±3.77a | 160.20±3.56 b |
| **Coir** | | 68.20 ± 6.51 c | 70.20±1.89 c | 130.20±4.12a | 243.50±1.87 a |
| **Perlite** | | 34.70 ± 4.58 b | 34.80±1.78 d | 46.10±2.33c | 38.30±3.22 c |
| **Rockwool** | | 345.10 ± 4.43 d | 210.30±3.12 a | 35.00±5.21b | 38.40±1.34 c |
| **Sheep wool** | **K** | 28.40 ± 3.16 a | 33.20±4.25 b | 12.20±2.11e | 11.20±0.45 d |
| **Peat** | | 115.70 ± 3.27 d | 86.30±3.11 a | 78.30±3.55 b | 86.30±2.11 b |
| **Coir** | | 113.20 ± 6.83 d | 83.10±5.23 a | 88.30±2.79 a | 98.30±1.93 a |
| **Perlite** | | 55.30 ± 0.59 b | 36.20±3.77 b | 44.30±2.11 d | 45.20±0.56 c |
| **Rockwool** | | 70.40 ± 0.32 c | 66.70±3.78 c | 55.30±2.56 c | 41.20±4.13 c |
| **Sheep wool** | **Ca** | 33.80 ±1.55 b | 34.10±2.77 c | 44.10±4.12 d | 55.10±2.13 c |
| **Peat** | | 64.90 ± 1.22 d | 71.20±3.21 a | 57.30±3.52 c | 89.30±1.23 b |
| **Coir** | | 55.70 ± 1.49 c | 67.20±3.33 a | 65.70±1.56 b | 88.70±0.14 b |
| **Perlite** | | 24.60 ± 1.30 a | 30.30±5.14 c | 32.30±0.45 e | 33.10±1.22 d |
| **Rockwool** | | 115.30 ± 3.00 e | 125.10±3.56 b | 112.40±1.32 a | 125.70±4.21 a |

The information of the table 5.39, conveys us the same accumulation of the nutrients as in example with the variants treated wit the biostimulating substances. It can be explained, as in previous example, by different nature of the substrates.

**Elements content in different plant parts of the cucumber plants.** Cultivation of cucumber plants on different horticultural substrates creates different conditions for development of the root system of the plant end thus can influence an uptake of the nutrient elements from both, nutrient solution and biostimulating mixture. Analysis of elemental content in different parts of the cucumber plants conducted to analyze a possible influence of *B.subtilis* FZB 24, LACTOFOL"O", K-Humate on the uptake of these nutrients. The plant specimens from the

treated variants (Figure 5.33) and without treatment (Figure 5.34) were sampled and tested for the content of Ca, K, P, Mg in leaves fruits and shoots of the cucumber plants.

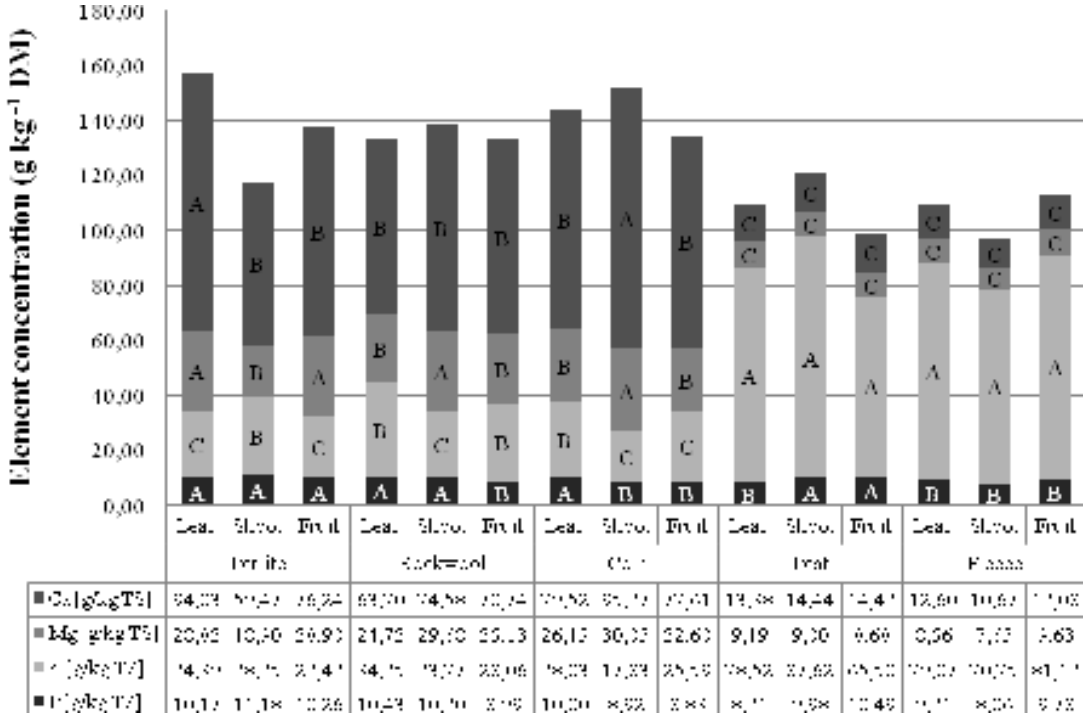


**Figure 5.33** Content of nutrient elements in different cucumber plant parts on the variants with biostimulating mixture. Different letters mean statistically significant difference. Two-way ANOVA. Tukey's HSD, $p<0.05$.

The results of the analysis and their statistical comparison showed that, the test plants on the treated variants with perlite, rockwool, coir accumulated high concentrations of calcium, whilst plant on peat and fleece tend to accumulate more potassium. Given the facts that all these variants were treated three times with a combination of *B.subtilis* FZB 24 0.2%+ LACTOFOL"O"0.1%+K-Humate 0.01%" in quantity of 300 ml, one can conclude that different accumulation patterns resulted from different nature (physical properties) of the substrates. Analyzing the elements content on the variants without treatment, opposite inference can be made. The cucumber plants on the variants with perlite, rockwool, coir and peat accumulated much lower amounts of Ca, but at the same time, higher concentrations of potassium, whilst the cucumber plants on the fleece proved to absorb more Ca and little K.

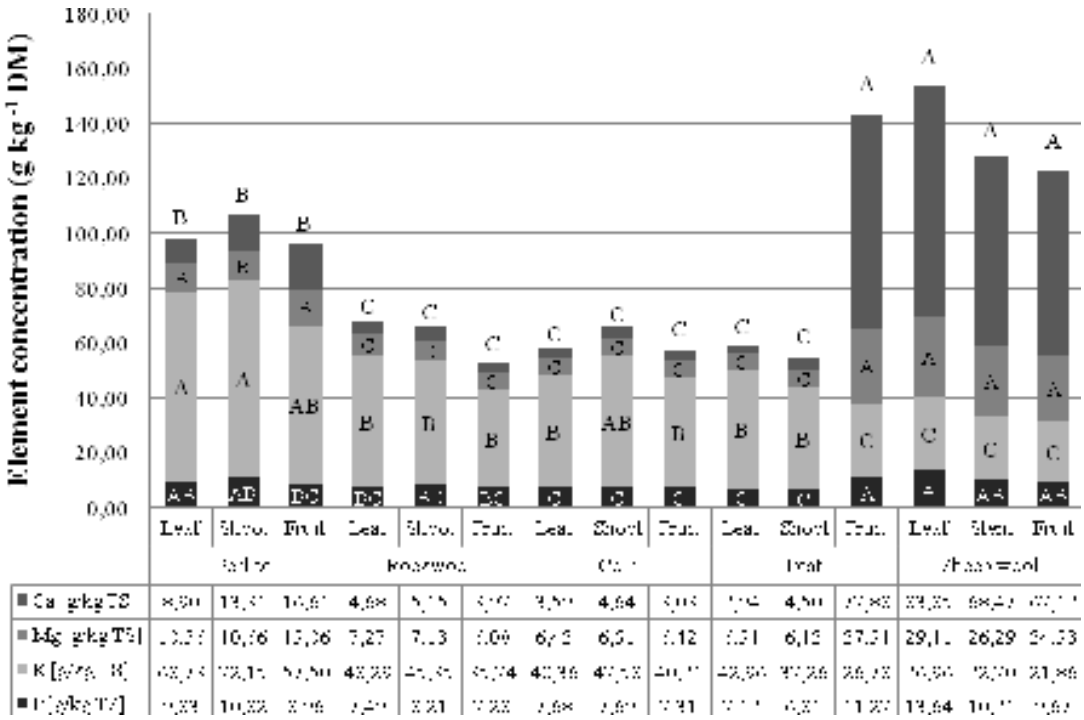


**Figure 5.34** Content of nutrient elements in different cucumber plant parts on the variants without biostimulating mixture. Different letters mean statistically significant difference. One-way ANOVA. Tukey's HSD, $p<0.05$.

The variant without treatment manifested an opposite trend with regard to accumulation of Ca and K. Accumulation of phosphorus on both variants remained on the same level and thus does not depend on substrates properties or treatment with biostimulating substances. Absorption of magnesium follows the same patters as in case of Ca but with lower concentrations. Treated variants with perlite, rockwool, coir and peat accumulated larger amounts of magnesium, whilst the variants without treatment manifested its lower concentrations.

**Discussion**

It can be stated that sheep wool requires a higher and more stable supply with nutrient solution than the other substrates. Nevertheless, in this experiment there was no problem with irrigation although there was no adoption of dripping frequency to this. Therefore sheep wool can be accepted for substrate culture. It should be investigated how long it takes to change the physical parameters of sheep wool to reach the target values. Maybe a pre-treatment with special washing solution can improve the physical parameters in the first time. In peat slabs there was also no or only a slight increase in the nutrient content from the first to the second vegetation. Only the accumulation of potassium was higher after the first vegetation. Regarding the high yield on

sheep wool and peat slabs after the second vegetation (Table 5.30) it can be assumed that the low content of minerals in the substrate resulted from the high uptake by the plant in these variants. At the same time lower water holding capacity of Sheep wool resulted in low nutrient binding capacity of nutrient elements.

The content of $N\text{-}NO_3^-$ and $Ca^{2+}$ in perlite was increasing during the second vegetation while the K content did not change. Concentration of these nutrients increased in thirds and fourth periods. The nutrients content in rockwool slabs was very high and should be discussed carefully. Maybe these values are due to the method how to take the samples. Nutrient solution of the rockwool was taken out with a syringe and analyzed; none of the elements were adsorbed. The yield (Table 5.30 and Table 5.31) of the first vegetation was lower than of the second and vegetation that followed, due to the differences in growing conditions for the first and second vegetation. Nevertheless also in the first vegetation the effect of substrates on the yield could be proved. In the standard variant (untreated) on peat slabs the yield was very low, sheep wool and rockwool had higher yields. By the application of the biostimulators (treated) the yield could be enhanced sometimes to the double but also after the treatment sheep wool and rockwool had the highest yields. In the second cultivation the yield was considerably higher in all variants. In this cultivation the highest yield could be recorded on sheep wool. The mineral substrates had much lower yields than the organic ones. But even under these good growing conditions the application of the biostimulators enhanced the yield.

### 5.3.2 Influence of the biostimulating mixture on the root length and biomass production

**Problem description**

In previous experiment, it was tested that application of lactates, humates, *B. subtilis* and their combinations as biostimulating substance brings better effects by root application. Biostimulating substance tested before consisted of three different biostimulators. It is not clear whether binary combinations (combinations of two substances) can evolve similar effects. At the same time, it can be assumed that application of binary combinations may have a narrower activity spectrum. This can have detrimental effect on development of cucumber plants.

The current experiment tested the application of Lactofol"O"+ *B.subtilis* FZB 24 and K-Humate+*B.subtilis* FZB 24 in comparison with control variant – without treatments and variant with combined biostimulating substance. Figure 5.35 illustrates the influence of these biostimulators on the quantity of cucumber leaves. The quantity of leaves on the control variants

comprised 25. This value does not show any statistical significant difference in comparison to variants with binary biostimulating mixtures. It is, however, does not reach values achieved on the variant with application of all three biostimulators. That can lead us to assumption that application of combined biostimulating substance induces formation of new leaves or delays senescence of the old ones.

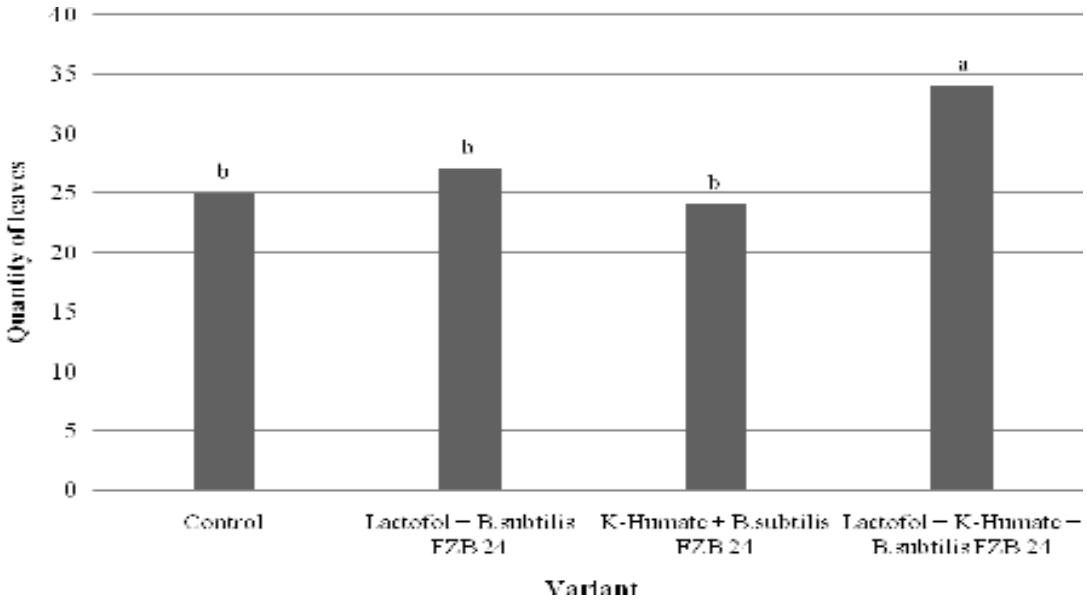


**Figure 5.35** Influence of different biostimulating mixtures on leaf quantity of cucumber plant. One-way ANOVA. Tukey's HSD $p<0,05$. Different letters mean statistical significant difference. n= 28

Application of biostimulating substances influenced the dynamic of leaves formation on all treated variants (Figure 5.36). The lowest leaf area was observed on control and Lactofol"O"+ *B.subtilis* FZB 24 and K-Humate + *B.subtilis* FZB 24. The difference between these variants to every single measurement date was not statistically significant. The same statement is valid for all measurements beginning from first week through 7-th week. Differentiation in leaf area dynamic can be observed beginning from 8-th week. Subsequent plants development differentiated values of the variant with application of Lactofol"O"+ K-Humate + *B.subtilis* FZB 24 – biostimulating mixture and other variants.

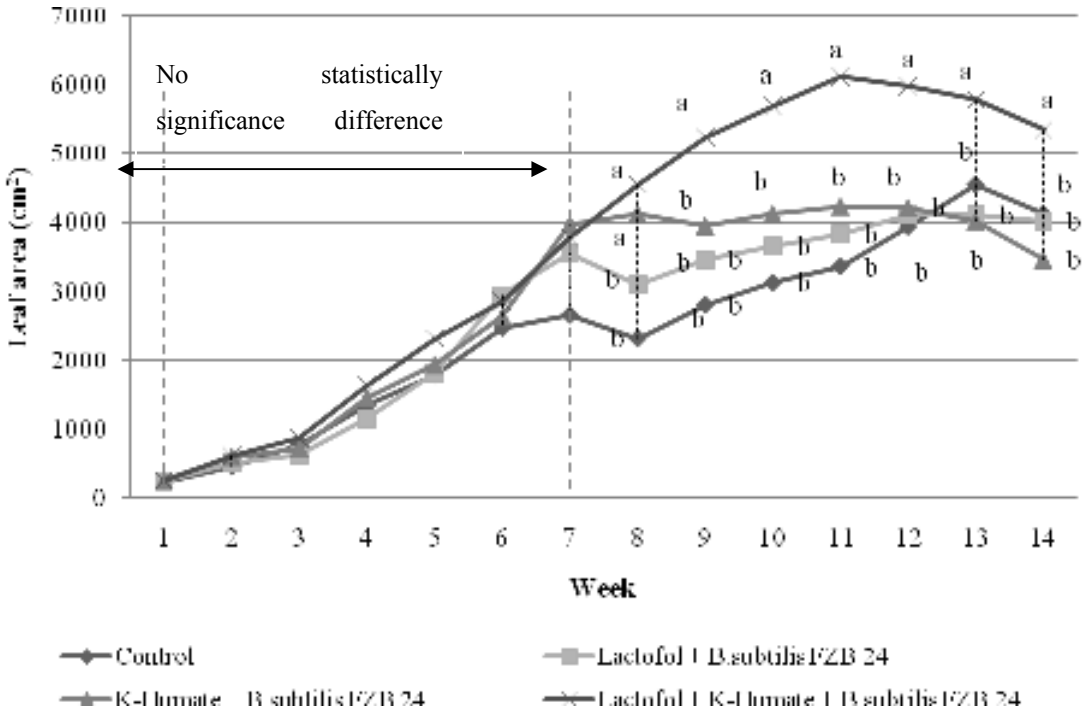


**Figure 5.36** Influence of different biostimulating mixtures on evolution of leaf area of cucumber plant. One-way ANOVA. Tukey's HSD $p<0,05$. Comparison only within measurement date. Different letters mean statistical significant difference.

This pattern remained unchanged to the end of the experiment. Application of biostimulating mixture in this experiment proved to increase leaf area of the test plants in the long run. Treatment were conducted three times on -1$^{st}$, 2-d and third week, but positive effects of it was observed only 5 weeks later (on 8$^{th}$).

The length of the stem does not always imply that it should have more leaves but at the same time, more leaves as a result of treatment can be accommodated on the longer stem. Apparently, the fact that biostimulators are applied to the root system of the plant produces its benefit by stimulating development of the root system of the cucumber plants, which subsequently results in higher phonological characteristics and productivity of the plants.

*B.subtilis* FZB 24, LACTOFOL "O", K-Humate had statistically significant effect on stem and root length of cucumber plants (Figure 5.37). An issue of optimality of growing conditions in the greenhouse can be addressed, as in this case, by application of biostimulating substances, those possess (as previous experiments show) a capability to relieve negative effects conduced on the horticultural plants. Previous researches showed that application of biostimulating mixture increases productivity of plants. The effects of the biostimulators observed on plant's growth included the influence on the yield formation, leaf area formation and stem length formation.

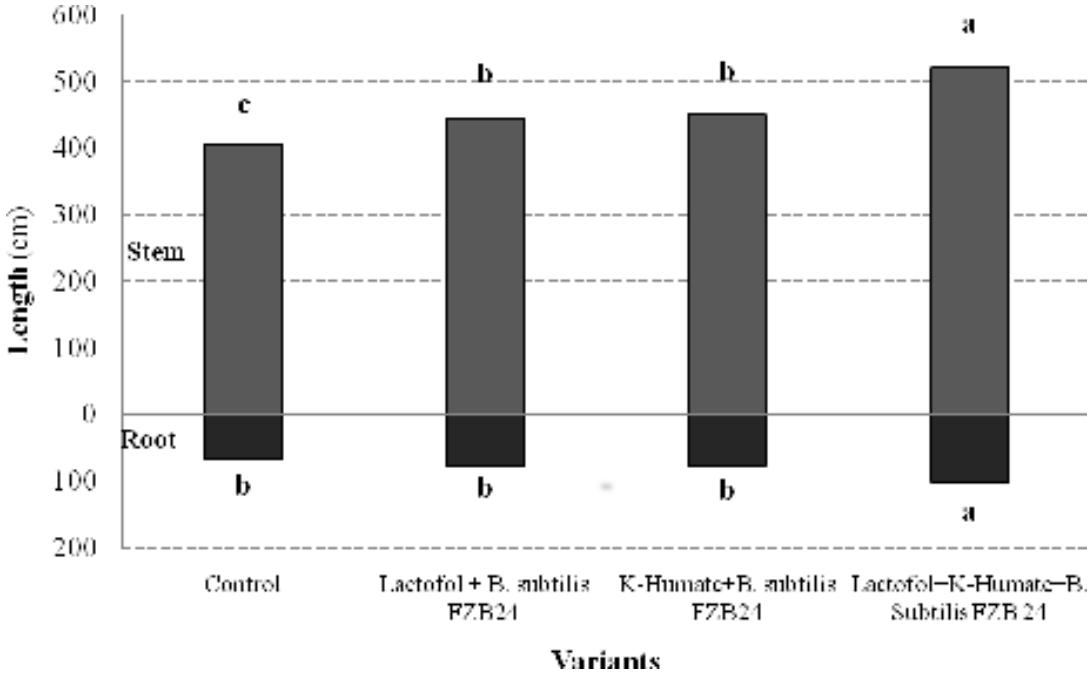


**Figure 5.37** Influence of biostimulating mixture (*B.subtilis* FZB 24, LACTOFOL "O", K-Humate) on length of roots and stems of cucumber plants. Tukey's HSD $p<0.05$. Different letters indicate statistically significant differences.

Formation of fruit yield cannot be sustained without extensive assimilation apparatus as well as developed root system. Experiment demonstrated that application of combined biostimulating mixture (*B.subtilis* FZB 24, LACTOFOL "O", K-Humate) has statistically significant effect on fresh matter of leaves and roots of cucumber plants. We can see that the variant with the mixture of three different biostimulating substances manifests higher fresh weight of roots –the fact that can play an important role in terms of nutrient supply of the test plants. In the experiment where combination of the treatments represent a factor that takes influence on the development of the test plants, it is shown that the application of the full biostimulating formulation supports the growth of the leaf area as it is the growth of the roots system of the cucumber plants (Figure 5.38).

Fresh weight of the specific parts of the plants is one of the characteristics that describe the growth processes (Figure 5.38). Fresh weight of the cucumber leaf and root followed the same pattern of the stem and root length. Application of the biostimulating mixture influenced the increase of the stem and root length of the test plants in the experiment

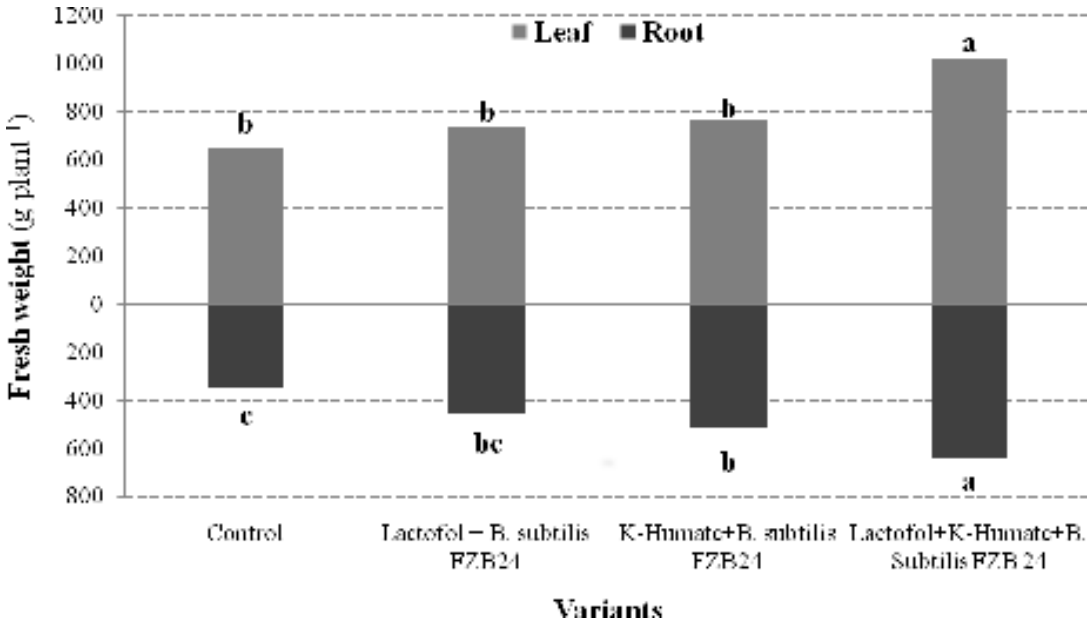


**Figure 5.38** Influence of biostimulating mixture (B.subtilis FZB 24, LACTOFOL "O", K-Humate) on fresh matter of roots and leaf of cucumber plants. Tukey's HSD $p<0.05$. Different letters indicate statistically significant differences.

The plants from this variant develop larger assimilation apparatus in comparison to all other variants. With regard to phonological characteristics of the cucumber plants, we can see that the same variant with all three compounds produced plants with longer stem as well as longer root system, which is in coincidence with information that about fresh weight of leaves and roots of the test plants. Plant productivity of the variants with combined biostimulating substance increases plants' productivity in comparison with other variants.

Different effects of the treatment were observed in quantities of the flowers and marketable fruits. Figure 5.39 illustrates number of the emerged flowers and number of the marketable fruits harvested during the experiment. The control variant had lowest quantity of both flowers and marketable fruits. This result does not have statistically significant difference in comparison to the variant with application of Lactofol "O" + *B.subtilis* FZB 24. Application of K-Humate + *B.subtilis* FZB 24 contributed to formation of more flowers on the cucumber plants and at the same time, increase of the marketable fruit quantity in comparison to the control variant. The variant with biostimulating mixture - Lactofol"O" + K-Humate + *B.subtilis* FZB 24 exhibits the highest quantity of the flowers and cucumber fruits (Figure 5.39). The gap between the flowers quantity and marketable fruits on this variant is the narrowest.

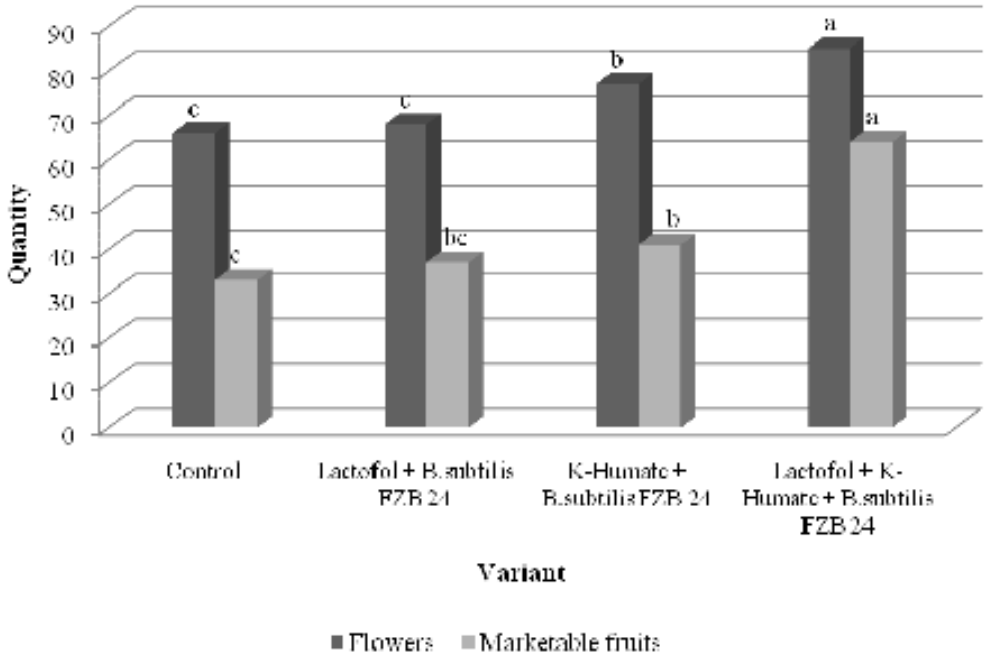


**Figure 5.39** Influence of different biostimulating mixtures on flowers and marketable fruits quantity of cucumber plant. One-way ANOVA. Tukey's HSD $p<0,05$. Different letters mean statistical significant difference.

It can be seen that application of the full biostimulating mixture directly influenced the transformation of the flowers of the cucumber plants into the marketable cucumber fruits. The yield of the test plants resulted from the previous characteristics of biological development of the cucumber plants (Figure 5.40). Although, not statistically significant, the variants with the binary combinations of biostimulating substances demonstrated lower productivity in comparison with plants on the variant without treatment (control).

Development of the root system coupled with the extensive development of the stem length and fresh weight of leaves (Figure 5.37) created biological background for formation of the fruit yield (Figure 5.40). The yield of the plants is statistically higher then on the other variants. Even under the growing conditions, often not optimal for the demands of cucumber plant, the application of full biostimulating mixture proved useful against the binary combinations or the variants without application of the biostimulating compounds (control).

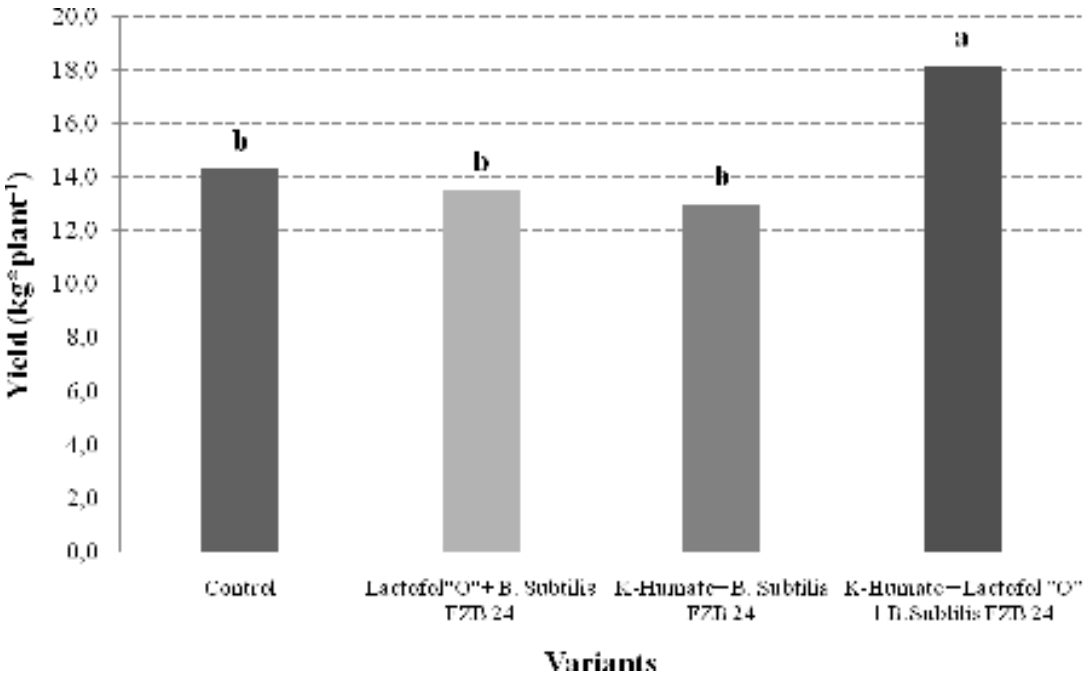


**Figure 5.40** Influence of different biostimulating mixtures on yield of marketable fruits of cucumber plant. One-way ANOVA. Tukey's HSD $p<0,05$. Different letters mean statistical significant difference.

Application of the combined biostimulating mixture has a statically significant positive effect on formation of green biomass of roots and stems of cucumber plants. The fresh matter of the roots and stems of the cucumber plants from the variant with application of the combined biostimulating substance (*B.subtilis* FZB 24 + LACTOFOL "O" + K-Humate) were significantly different from other variants. Application of the binary combinations (*B.subtilis* FZB 24 +K-Humate; *B.subtilis* FZB 24 + LACTOFOL "O") had no statistically significant effects on stems' weight. The plants from the variant without application of the biostimulating mixture (Control) did not show statistically significant difference in the root fresh weight with variant *B.subtilis* FZB 24 + K-Humate. The conclusion of the experiment - *B.subtilis* FZB 24 + LACTOFOL "O" + K-Humate helped to increase the productivity of the cucumber plants. Analysis of elemental content of different parts of cucumber plants is presented in the figure 5.41.

Use of the standard nutrient solution in this experiment contributed to the balanced supply of the cucumber plants with the necessary nutrient elements. Since conditions of the experiment were optimal, there are no drastic fluctuations in the mineral content in the different parts of the cucumber plants.

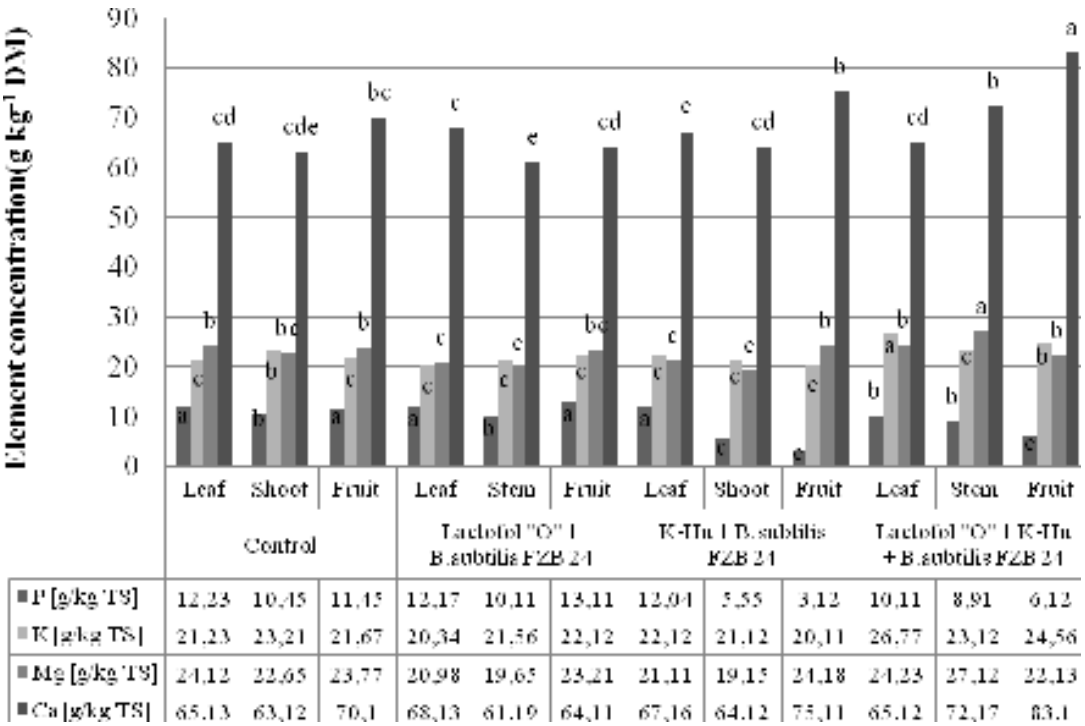


**Figure 5.41** Influence of different biostimulating mixtures on flowers and marketable fruits quantity of cucumber plant. One-way ANOVA. Tukey's HSD $p<0,05$. Different letters mean statistical significant difference.

It is possible point out that emerged statistically significant differences in the elemental content of the different parts of the cucumber plants can be attributed to the composition of the biostimulating mixtures being applied in the experiment. Since the plants were cultivated on the same substrate (perlite) and supplied with the same standard nutrient solution it is on the content of biostimulating mixture (twofold or triple) to influence content of nutrients. It is seen from the figure 5.41 that application of all three biostimulating substances contributed to highest content of phosphor in the fruits of cucumber plants. The highest content of the magnesium was accumulated in the stem of the plant from the same variant.

**Discussion**

Every constituent part of the biostimulating mixture plays a role in the formation of the growing conditions that influences the development of the cucumber plants. In the course of the vegetation experiments, primarily three-component mixture of the biostimulators was tested – *B.subtilis* FZB 24 0.2% + K-Humate 0.01% and *B.subtilis* FZB 24 0.2% + LACTOFOL "O" 0.1%. The question of better efficacy of these substances can be answered through comparison of the binary combinations of the biostimulators. It was established that a biostimulating mixture,

in general, stimulates development of the plant organs and creates optimal conditions in the rhizosphere, which enables plants to develop longer root systems (Figure 5.37) which can supply the plants with macro- and micronutrients over the period of vegetation (Figure 5.41). The application of the binary combinations did not show statistically significant changes in terms of formation of the marketable fruits of the cucumber plants (Figure 5.39). This fact is subsequently reflected in comparison between the variants with the binary applications of the biostimulating substances and the full formulation of the biostimulating mixture (Figure 5.40). It is proved that the binary combinations of the biostimulating substances are less effective.

The comparison of the different combinations of the biostimulating substances in this experiment demonstrated that application of all biostimulators in formulation Lactofol "O" 0.1% + K-Humate 0.01% + *B.subtilis* FZB 24 0.2% is the best choice in comparison to the binary mixtures - *B.subtilis* FZB 24 0.2% + K-Humate 0.01% and *B.subtilis* FZB 24 0.2% + LACTOFOL "O" 0.1%.

### 5.3.3 Effect of the biostimulating mixture under abiotic stress conditions

Problem statement.

Cultivation of the horticultural plants in the greenhouse conditions gives the benefit of the control over most growing factors that are involved in the production. Nevertheless, the occurrence of suboptimal growing factors cannot be excluded. The suboptimal values of pH, EC, temperature are abiotic stress factors that can limit productivity of the horticultural crops. Vast portion of the biostimulators can be applied as stress relieving agents. In this case, any effect of their application can be expected during the phases of suboptimal environmental conditions. Such conditions are subdivided into biotic and abiotic factors.

The experiment tests the hypothesis that the biostimulating mixture of K-Humate, LACTOFOL"O", *Bacillus subtilis* FZB 24® has stress relieving effect on the plants that undergone periods of suboptimal growth conditions. Chlorophyll-*a* fluorescence was used for evaluation of the physiological effects occurring in the cucumber plants especially under the influence of the suboptimal growth factors. Three separate experiments were conducted. This experiment analyses the influence of suboptimal growing factors on the changes in the photosynthetic capacity of the cucumber plants with and without treatments with biostimulators. The mixture of the three different watery solutions of K-Humate 0.1%, LACTOFOL "O"0.2%, *Bacillus Subtilis FZB24* 0.2% was used for the treatments.

Because of the limited volume capacity of the climate chamber, the cucumber plants were removed from it before they were able to develop any marketable fruits. Many factors such as stage of the plant's developmental combined with presence of biotic and abiotic suboptimal factors (stresses) can reduce photosynthetic efficiency of the photosystem II of the horticultural crops and therefore, their decrease their productivity. The chilling stress is being applied to the cucumber plants for the duration of three hours triggered a sharp decrease of Fv/Fm parameter (Figure 5.42).

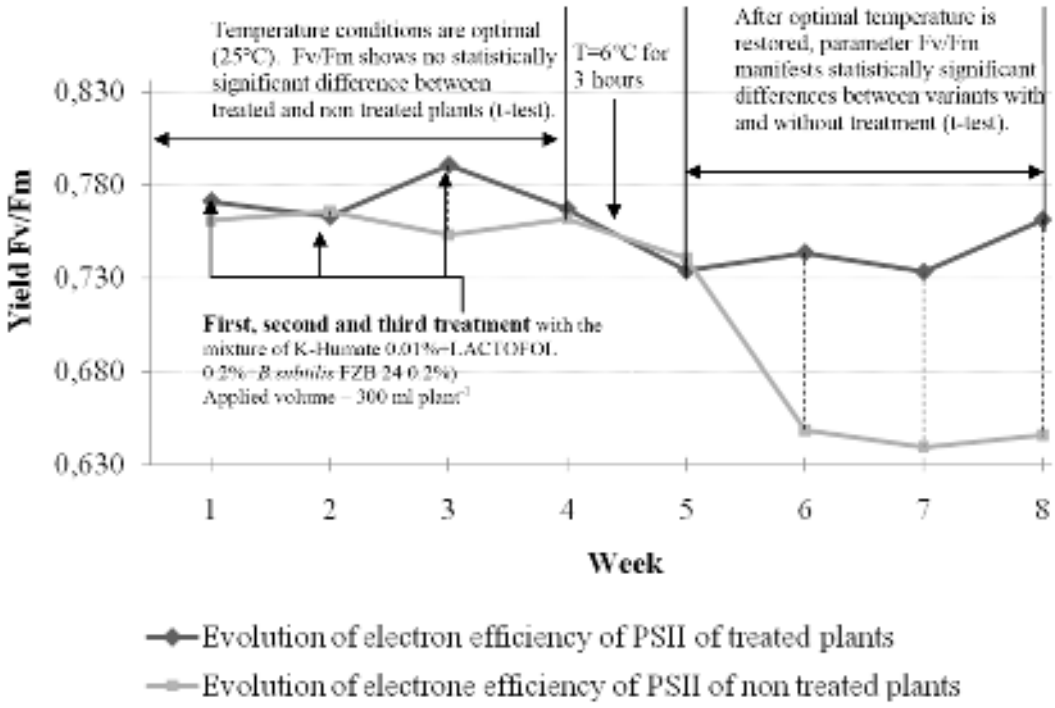


**Figure 5.42** Electron efficiency of photosystem II of cucumber plants (Temperature stress)

The results show that the photosynthetic capacity of the cucumber plants did not change significantly, despite the fact that the half of the test plants was treated with biostimulating mixture. During the first four weeks after transplantation to Mitscherlich pots, plants do not show any statistically significant difference with regard to the electron efficiency of the photosystem II. Application of the biostimulating mixture did not influence photosynthetic status of the treated plants. It can be observed that the increase of Fv/Fm during the third measurement was not statistically different from the data obtained on the variant without treatment.

Application of the chilling stress between 4-th and 5-th measurement of electron efficiency of the photosystem II, for the duration of 3 hours, still did not show any significant differences in value of Fv/Fm although it did signified a downward trend in the quantum yield of PSII.

Subsequent restoration of the optimal air temperature conditions to the level of 25°C did little to stabilize photosynthetic apparatus of the non treated cucumber plants.

During the last 3 measurements the values manifested significantly low levels of Fv/Fm and it remained unchanged through the rest of the vegetation. The treated plants, however, recovered their photosynthetic potential during the last three weeks of the experiment. The values of the quantum efficiency of PSII did not drop below 0.730 and during the last measurement the recorded, on average, 0.775.

Admittedly, the levels of electron efficiency of PSII of both, treated and non treated plants, remained well below an optimal level of at least 0.780. In the practical terms, it means that plants that were exposed to such drastic temperature decrease, even for relatively short period of time, would not be able to recover their economic potential, nor is it possible under industrial greenhouse conditions. On the other hand, as it has already been described in the early literature review chapters, many biostimulators evolve their biostimulator activity only under the suboptimal growing conditions. The experiment with suboptimal pH values was conducted to test this theory (Figure 5.43).

The experiment shows the same pattern in development of the photosynthetic activity of the photosystem II as the previous one. The measurement of Fv/Fm was conducted weekly. The difference is that reduced pH values 3.3-3.5 were in place for a week. The reading of the quantum yield during first for weeks did not exhibit any significant changes and shows sustainable, upward trend, which is typical for the plants being transferred to a new substrate. It can be pointed out that the kind and level of the stress is always of the essence. It is also important to notice the difference between the time of the particular stress condition and its protraction over the vegetation period of the cucumber plants. The reduction of pH values to the level of 3.3-3.5 significantly influenced the electron efficiency of PSII between treated and non treated variants. The reduction of Fv/Fm vales were less steep, in fact, 5-th and 6-th measurements are in the same region as values before the stress conditions. The reaction of the cucumber plants on the variant without treatment was much explicit in terms of the electron efficiency reduction of PSII. Fv/Fm values of non treated plants during the 5-th measurement showed significant reduction to the level of 0.650. During the 6-th measurement the values of Fv/Fm dropped further and showed on average 0.635.

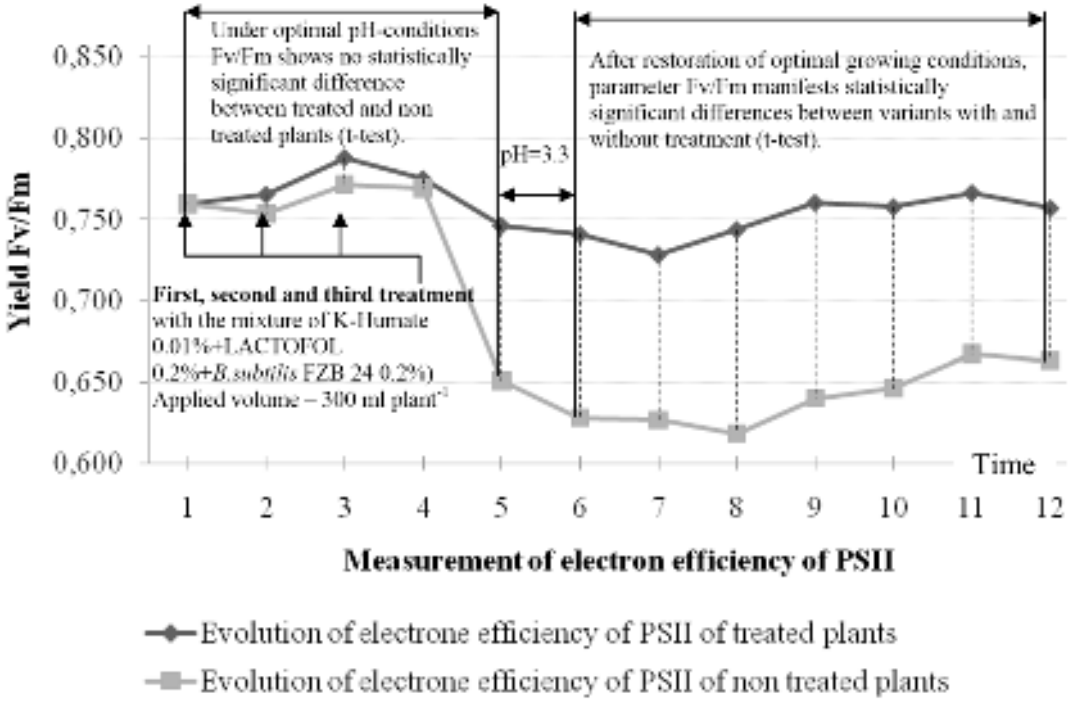


**Figure 5.43** Evolution of electron efficiency of photosystem II of the cucumber plants being exposed to suboptimal pH-condition (acid)

The photosynthetic efficiency of the plants depends on the stage of development of the plant and presence of the biological and physiochemical stresses. In addition, each step of the photosynthetic process from the absorption of the light energy to the conversion and storage of the energy in the synthesis of the sugar molecules can be affected differently by various limiting factors. As in this case, pH values of the nutrient solution can vary strongly. The practical experiences show, that the reaction of the nutrient solution tends to change in alkali direction (VERDONCK and GABRIELS, 1988).

The occurrence of the alkali conditions in the rhizospheric area during the vegetation period can cause disruption in the nutrients uptake by the plants, and therefore, decrease the yield in quantity and quality. The influence of the biostimulating substances on the cucumber plants was tested under simulated alkali reaction of the nutrient solution (Figure 5.44). At the beginning of the experiment the cucumber plants showed the highest electron efficiency of PSII of all. This statement is true for both treated and non treated plants of the experiment.

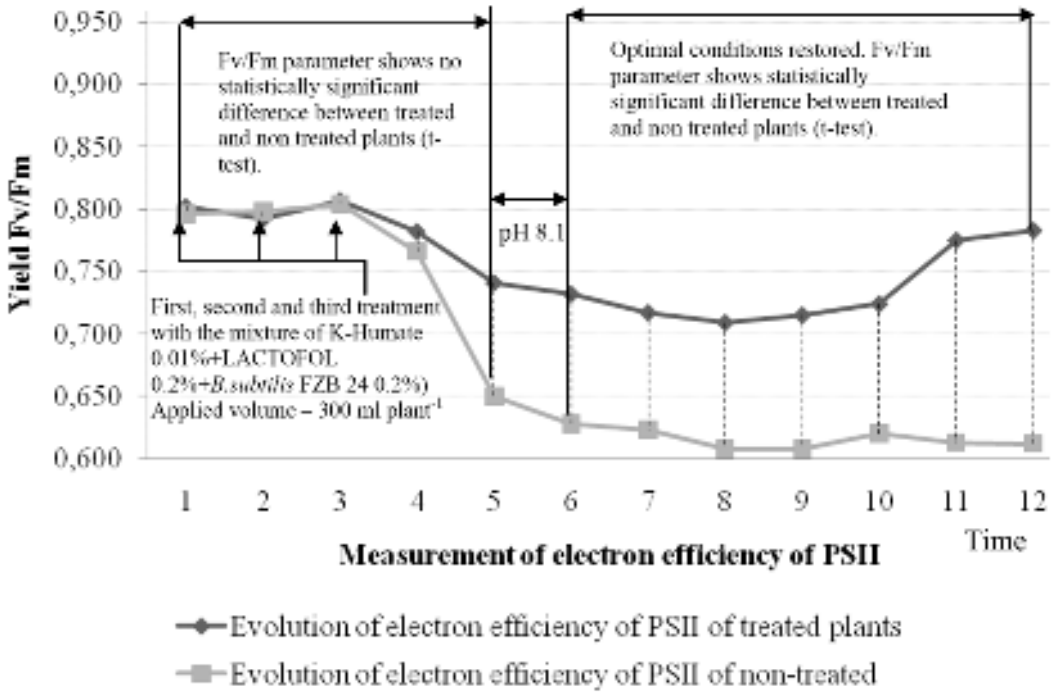


**Figure 5.44** Evolution of electron efficiency of photosystem II of the cucumber plants being exposed to suboptimal pH-condition (alkali)

The first statistically significant change of Fv/Fm values was registered during the 5-th measurement which coincided with onset of stress phase of the experiment. The subsequent development of the electron efficiency of the photosystem II of both, treated and non treated plants, remained significantly different. The beginning of the 10-th measurement, Fv/Fm values of the treated plants started to grow, and during the 11-th and 12-th they showed comparable results with the values before application of the stress factor.

The cucumber plant is very demanding with regard to growing conditions. The sensitive reaction of the photosynthetic apparatus of the cucumber plants to changes in the values of different growing factors found its reflection on subsequent changes of plants phenology (Figure 5.45).

The exposure to suboptimal growing conditions during the first weeks of the vegetation can have a deleterious effect on the entire productivity of the plants. Application of the biostimulating mixture during the first several weeks (before blossoming) proved to have positive effects on the cucumber plants exposed to the suboptimal abiotic growing factors (temperature and pH values of nutrient solution).

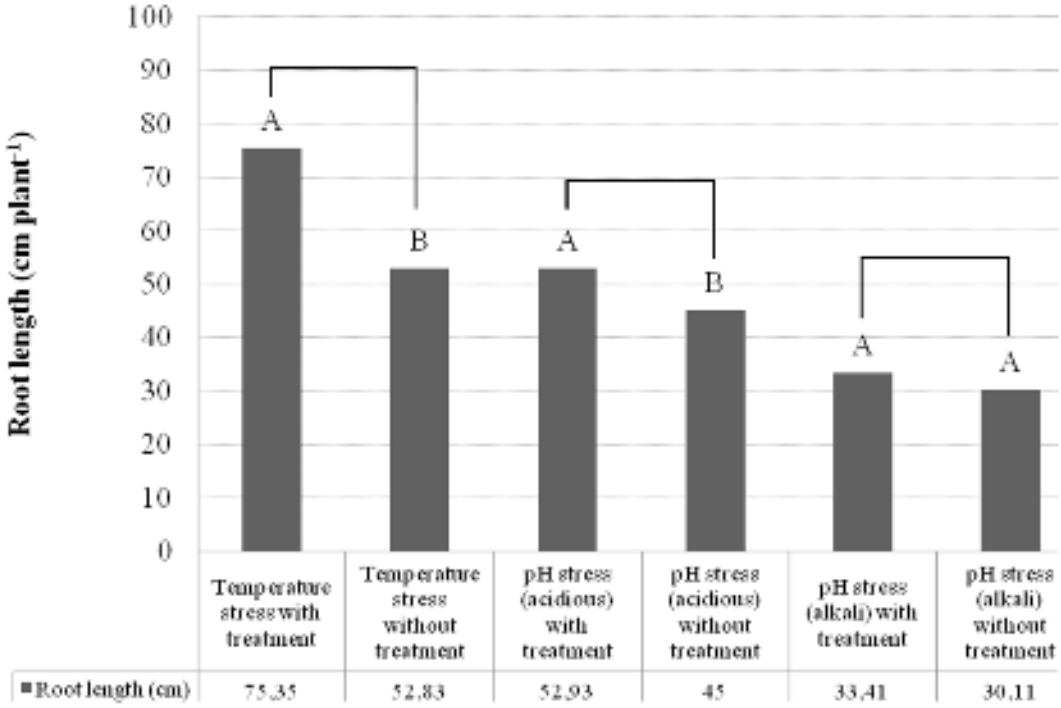


**Figure 5.45** Influence of suboptimal growing conditions on the root length of the treated and non treated cucumber plants**.** Statistics: t-test for pairs of variants with the same suboptimal growing factor. n=40

Considering the functioning of the photosynthetic apparatus of the test plants, it is necessary to compare the biological development of the leaf area (Figure 5.46). The same differentiation pattern, with regard to the leaf area, was preserved for the first two experiments. The leaf area showed statistically significant differences in all experiments on variants with treatment. As it is in examples with the treatments in previous experiments, the influence of the biostimulating mixture consisting of K-Humate 0.1%, LACTOFOL "O"0.2%, *Bacillus Subtilis FZB24* 0.2% can be effective during occurrence of the suboptimal growing conditions. The plants react to the treatments by changes in their physiological development that finds its expression in the root system growth, leaf area dynamics and ultimately the yield of the fruits.

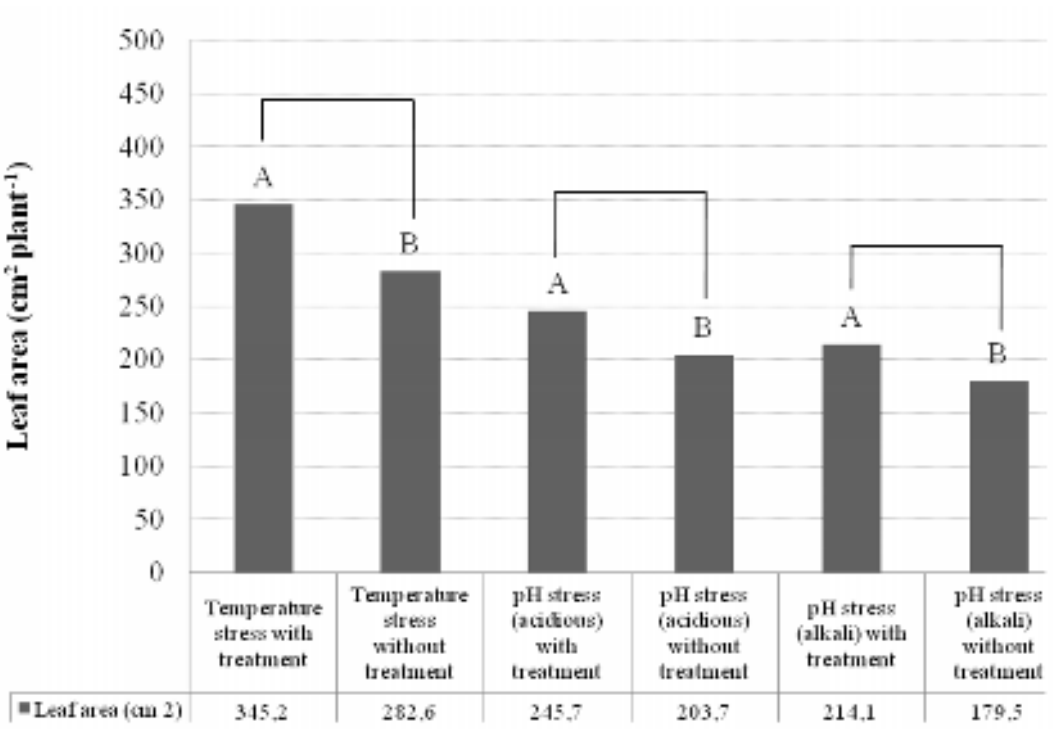


**Figure 5.46** Influence of suboptimal growing condition on the leaf area of cucumber plants
Statistics: t-test for pairs of variants with the same suboptimal growing factor. n=40

The air temperature, in the climate chamber, as a stress factor, differently influenced the root length of the cucumber plants with and without treatment. The statistically significant differences in the root length were noticed in the experiment with acidulous pH values. In the both experiments, the treated variants exhibit longer root length, which let us to conclude, that combination of humate, lactate and *B.subtilis* possesses stress relieving characteristics. Combined with values of the electron efficiency of PSII, the root values of the test plants are indicators of the stress protective activity of the biostimulating mixture. The conclusion from this experiment – treatments with biostimulating mixture contribute to formation of leaf area of cucumber plants. At the same time, the leaf area values are different in different experiments despite the same treatments.

Having considered different effects conferred on the plants by suboptimal growing conditions, it can be considered that the relation between the different plants parts can be affected by the treatments (Figure 5.47). The regression analysis was performed between the mass of the root system of the plants and their green biomass. The green biomass, in this case, is aboveground vegetation of the cucumber plants. The root length and the leaf area of the plants in treated variants were higher than in those without treatment (Figure 5.45 and Figure 5.46). The treated plants under the temperature stress developed higher root length. The root length in experiment

with pH stress was less expressed. The treated plants in both pH- and temperature stresses show very close relationship between the green biomass and mass of the roots.

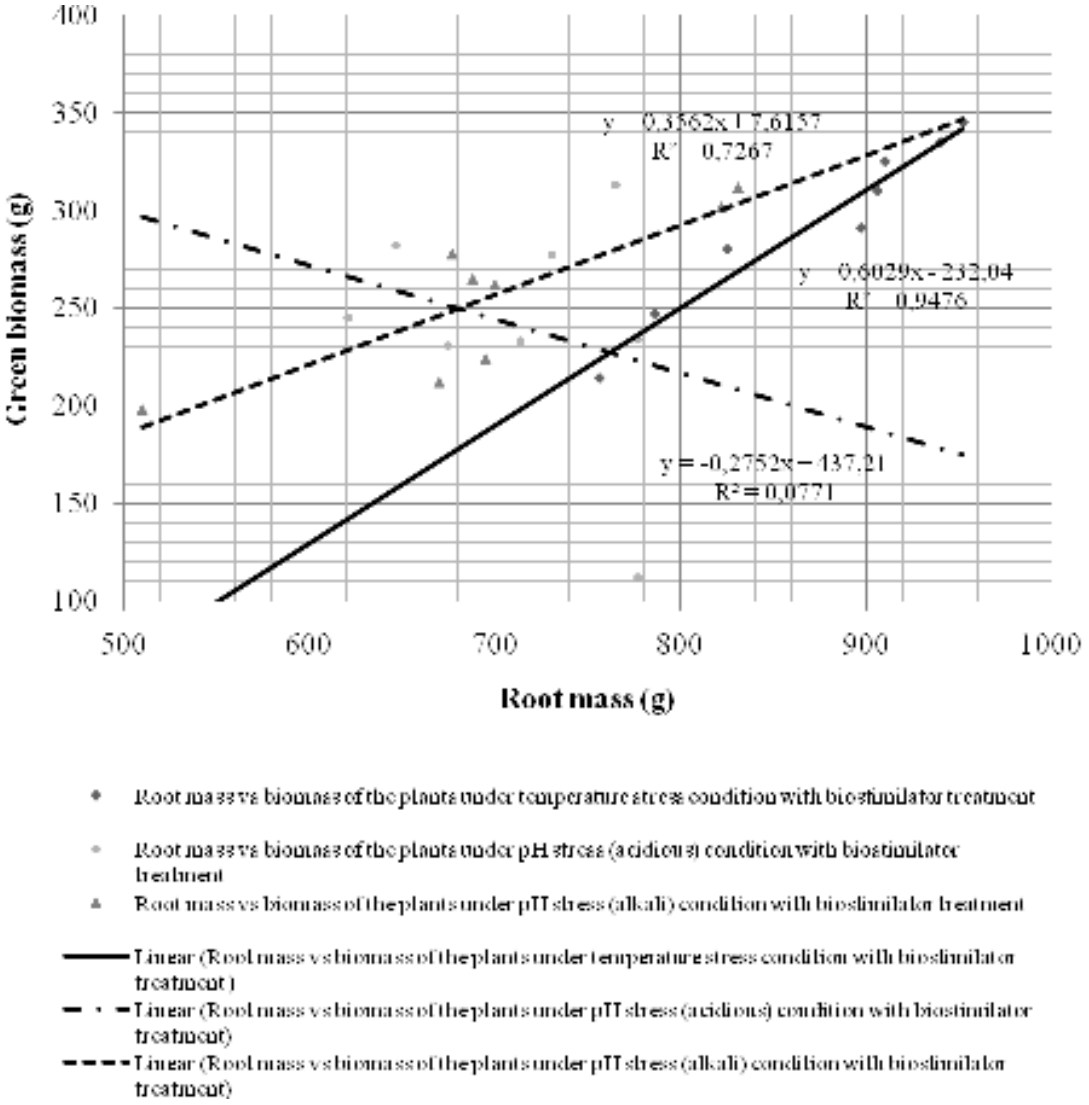


**Figure 5.47** Green biomass vs. root mass of the cucumber plants under different growing conditions. Pearson's correlation.

$R^2$ linear = 0.949 (Figure 5.47). This close relationship leads to the conclusion that the increase in the root mass is connected to the formation of the larger stem and leaf mass of the test plants. That is opposite to the plants without treatment where $R^2$ linear = 0.077 (Figure 5.47). Developing similar patterns of Fv/Fm in both temperature and pH-stress conditions one can conclude that application of the biostimulating mixture is capable to relieve plants during the period of the vegetation marked by suboptimal abiotic factors. The combination of K-Humate (0.01%), LACTOFOL"O" (0.1%) plays an important role in the increasing root length while *Bacillus subtilis FZB 24* can invoke the defensive mechanisms of the plants.

Thus, plants have an ability to absorb water and nutrient elements which result in higher stem and leaf mass. Application of *Bacillus subtilis FZB24* (0.2%), K-Humate (0.01%), LACTOFOL"O" (0.1%) proved useful in mitigating the stress impact on the cucumber plants. The parameter of the electron efficiency of the photosystem II showed the robust increase in the after-stress period. The cucumber plants with no treatment showed very low Fv/Fm values implying that they were not able to recover from temperature- as well as pH-stress.

The results show that there is strong correlation between the green biomass of treated cucumber plants and their root mass. The opposite of those treated plants, the variant without treatment does not show that dependency. Indeed ($R^2$ linear = 0.077) in the experiment with acidulous pH reaction showed very weak correlation. Application of the biostimulating mixture makes difference more when it comes to the temperature stress and it increases the root mass of the plants regardless of the stress factor in this experiment.

**Discussion**

The air temperature, as a stress factor, influenced the test plants to a lesser extend then pH values. That discrepancy can be explained by the fact that the stress factors are different by their activity spectrum and their nature. For instance, the changes in pH values of the nutrient solution contribute to the reduction in the nutrients uptake by the test plants, at the same time, the changes in the temperature conditions has no direct influence on the plants ability to uptake nutrient elements. Another factor – is the time during which a particular stress factor being applied. In the experiment with the temperature as a stress factor it was applied for the duration of 3 hours, at the same time, application of the different pH values as suboptimal growing factors were in effect for a one week. Apparently, if the temperature condition of 3°C was in effect for the same period of time as pH values, then the test plants would not survive. Adjustment of the temperature and pH values to suboptimal levels - stress factors were applied to test plants' responses and reactions on the level of the photosystem II, the electron efficiency of which changes in response to suboptimal environmental factors. After transplanting, plants, in experiment with pH stress, development of Fv/Fm parameter showed the upward trend at the beginning from 0.760 and up to 0.790 for plants with treatment and 0.770 – plants without treatment. The drastic decrease in the electron efficiency was observed after application of pH stress. Between the 4-th and 5-th measurements Fv/Fm of the treated plants fell to 0.747 and non-treated to 0.654. These values can be described as such of the plants exposed to suboptimal factor(s). The lowest Fv/Fm values for the treated and non-treated plants were 0.730 and 0.620

respectively. In the after stress period treated plants managed to recover to the larger extend (0.765- max. Fv/Fm value). The electron efficiency of the non-treated plants remained very low 0.670. In practical terms it means that the treated plant would be able to bring at least some marketable fruits which would not be the case in the other variant. The temperature stress was applied by decreasing the air temperature in the climate chamber right after the third treatment by the biostimulating mixture. The Fv/Fm parameter showed the same pattern as in the case of pH stress (Figure 5.44). Under the temperature and pH stresses, the Fv/Fm values tumbled down considerably indicating the reduction in the photosystem II efficiency. After the stress, only plants with the treatment were able to reach higher levels of Fv/Fm 0.662. That fact allows to conclude that the application of the biostimulating mixture at the beginning of the vegetation period of the cucumber plants tends to evolve the stress relieving functions under the suboptimal growing conditions.

### 5.3.4 Investigation of microbiological activity of substrates

**Problem description**

Application of the biostimulating mixture in the previous experiments manifested its efficiency in relieving consequences of the suboptimal growing conditions. At the same time, formulation of the biostimulating mixture contains substances that promote development of the microflora in the horticultural substrates. The previous description of the properties of K-Humate and LACTOFOL"O" implies that this substances can serve as the additional substrate for the colonies of microorganisms. The biostimulating mixture contains also *B.subtilis* FZB24.

The objective of this experiment is to test microbiological activity of the horticultural substrates during the long term utilization. The horticultural substrates were used for cultivation of the cucumber plants. The half of the substrates in combination with application of the biostimulating substances represents the treated variants. The second half of the experiments with the same substrates and the same test plants represents variants without treatment. The microbiological activity of the horticultural substrates was determined using the substrate induced respiration (SIR) method.

Since the method itself represents here the result of the experiment, it is described in this section. The method of the substrate-induced respiration is generally applied in the soil analysis. The method is based on the findings that soil/substrate associated microflora responds to introduction of glucose with immediate increase of the respiration. In this research the method is applied in order to determine activity of the substrate associated microflora. Addition of glucose to

stimulate growth of the microbial community results in efflux of $CO_2$ that is used for judgment on microbial activity of the substrates. In general, the optimal concentration should provide the best response in terms of $CO_2$ efflux (Figure 5.48).

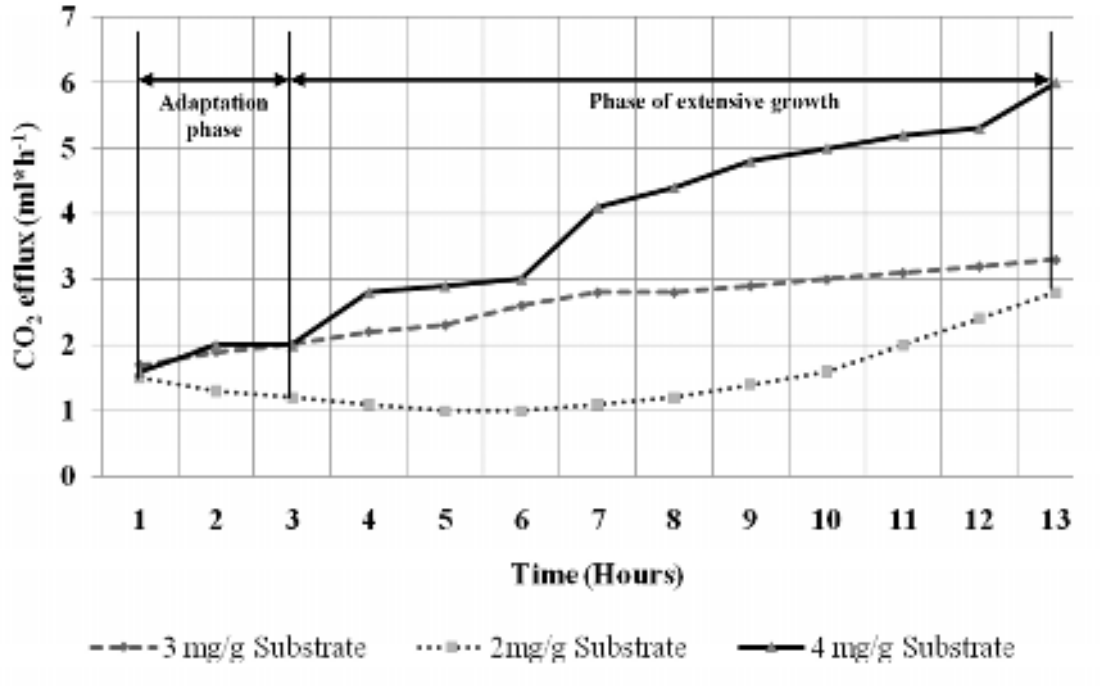


**Figure 5.48** Experimental determination of optimal glucose concentration on different horticultural substrates

This concentration provides the best response of the microbial community and efflux of $CO_2$ after adaptation phase starts to grow more rapidly. The approach to interpretation of the microbial activity in the substrates ($CO_2$) is based on the $CO_2$ efflux over the certain period of time. Recorded data of $CO_2$ efflux during 13 hours of incubation are used to evaluate microbial activity of substrates. Addition of glucose has caused growth of microbial biomass which resulted in the incremental growth of the substrate respiration (Figure 5.48).

Integral $CO_2$ efflux data, over the period of time is used for the evaluation of the differences in respiration between the variants. The trend function is built, according to the production of $CO_2$ vs. time of the analysis. In figure 5.49, shows theoretical approach to evaluation of the integral microbial activity of the horticultural substrates with and without impregnation with glucose. The marked area of the graph is an incremental growth of $CO_2$ efflux. The upper boundary of the integral calculus, which is an end of the adaptation phase (***a***) and the lower boundary (***b***) is a 13 hour time boundary. The same approach is adopted for determination of the basal respiration (respiration without glucose addition).

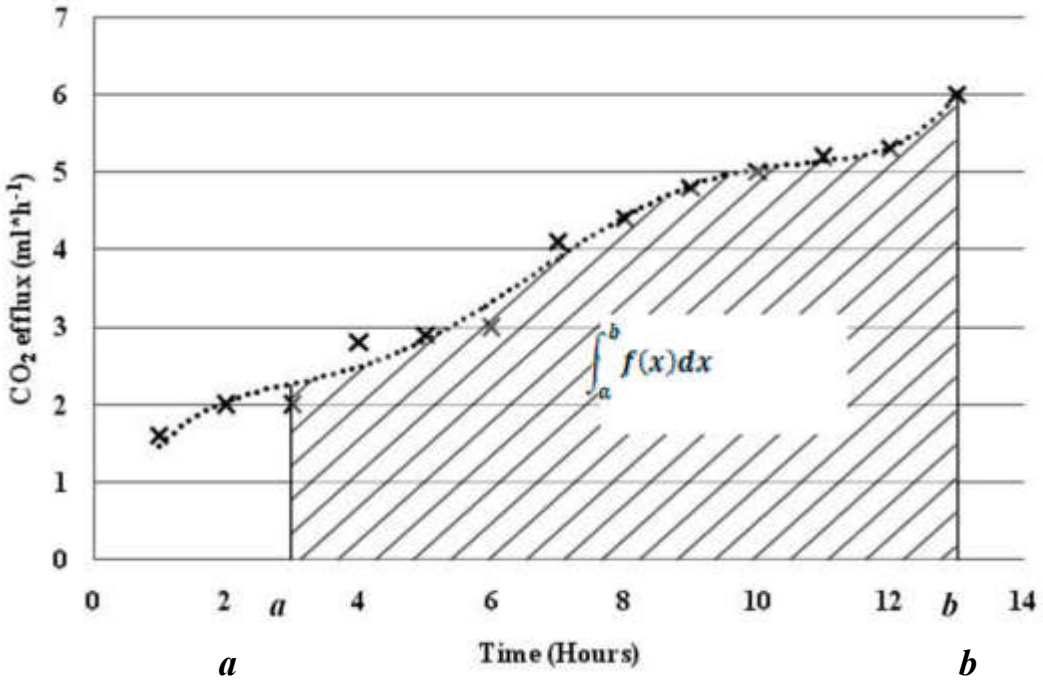


**Figure 5.49** Calculation of integral $CO_2$ efflux, during active growth phase of microbial community, using polynomial trend curve

"Other inputs" are considered as treatment of the variants with combined biostimulator (*B.subtilis* FZB24, LACTOFOL "O" and K-Humate). Being applied on the substrates (perlite, rockwool, peat, coir, sheep wool) it contributes to formation of the specific microbial community through introduction of *B.subtilis* FZB24. Microbiological activity presented as $CO_2$ efflux is used to quantify microbiological status in the substrates.

Basal respiration (Figure 5.50) represents $CO_2$ efflux from the microbial community of the substrate without addition of glucose. Total volume of $CO_2$ efflux within certain period of time (which is individual for every substrate) is interpreted as integral respiration.

Integral respiration is being calculated as integral calculus of trend function which in turn is determined as trend line of $CO_2$ efflux increase. Detailed calculation is described in attachment 1.

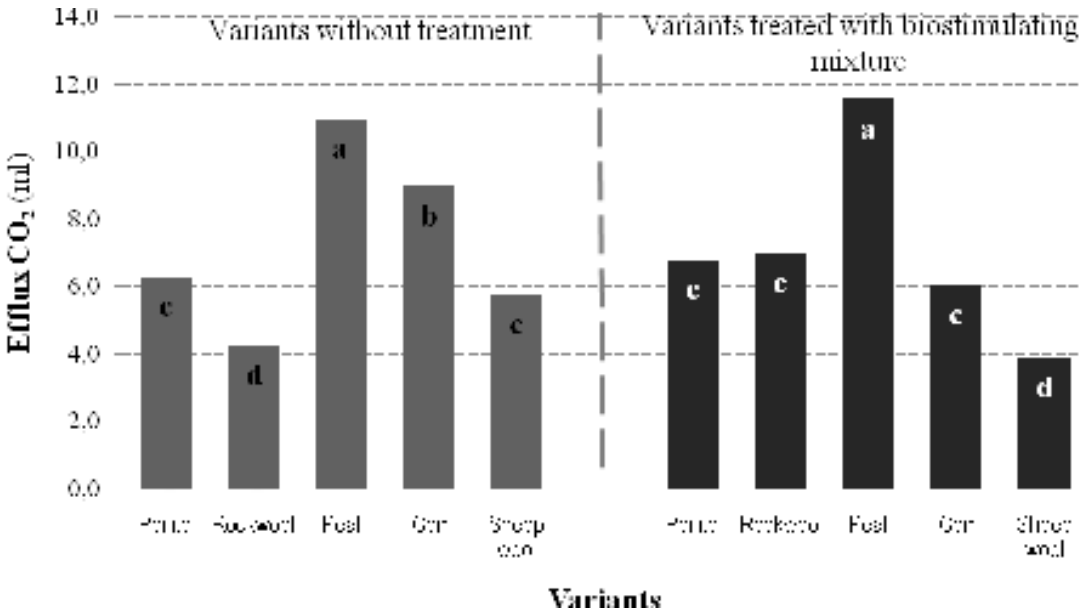


**Figure 5.50** Integral basal respiration of the substrates. Tukey's HSD $p<0.05$. (Different letters indicate statistically significant differences).n=30

Addition of certain (optimal) amount of glucose to the substrates triggers glucose-induced respiration (Figure 5.51). Glucose is used as a preferable source of energy for microorganisms and creates conditions for the respiration – a sign of the active metabolism of the microorganisms. The basal and glucose-induced respirations as function of microbial activity in the substrates inevitably interact with the root system of the plant. Depending on composition of the microbial community as well as its metabolic activity influences the rhizosphere of test plants. The detailed calculation of integral glucose-induced respiration of the substrates is shown in the attachment 1.

Relationship between productivity of cucumber plants is investigated using Pearson correlation (Figure 5.52 and Figure 5.53). It shows interconnection between plant's productivity and integral $CO_2$ respiration.

Substrates in this experiment possess different physical (BÖHME *et al.,* 2007) and chemical properties. Half sample of each substrate was treated a mixture of *Bacillus subtilis* (FZB24), Lactate (LACTOFOL®) and K-Humate during the vegetation period. The yield was higher in organic and treated substrates than in mineral and non-treated ones (Table 5.7.2). This could be due to the different microbiological status of the treated and non-treated substrates.

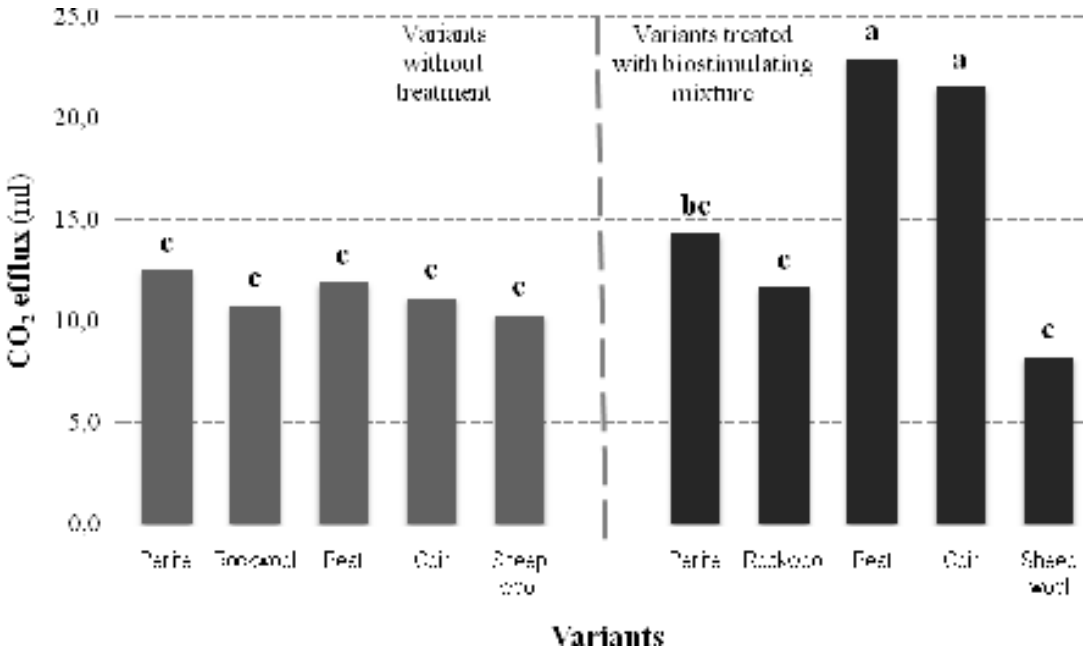


**Figure 5.51** Integral glucose-induced respiration of the substrates. Two-way ANOVA Tukey's HSD **p<0.05**. (Different letters indicate statistically significant differences). n=30

Because so far the SIR method is only used for soil analysis, the experimental system regarding glucose concentration used had to be adapted for horticultural substrates. It was experimentally established that 4mg glucose per g substrate$^{-1}$ can be used with all substrates to determine glucose-induced respiration. The integral basal respiration was highest for peat in treated and non-treated treatments (Figure 5.52) indicating a higher microbial activity in this substrate. Between treated and non-treated treatments of the same substrate there was no difference in this parameter. Therefore, it can be assumed that the microbial activity is not considerably increased by application of the biostimulators, but the composition of the microbial community might be changed. Determination of the integral glucose-induced respiration showed no differences between the substrates in the non-amended treatments but statistically significant differences between organic substrates (peat and coir) to other substrates in the amended treatments. The values for the glucose-induced respiration in the amended substrates were enhanced in comparison with non-treated not only in peat and coir but also in perlite. For rockwool and sheep wool this parameter was no different in treated and non-treated substrates.

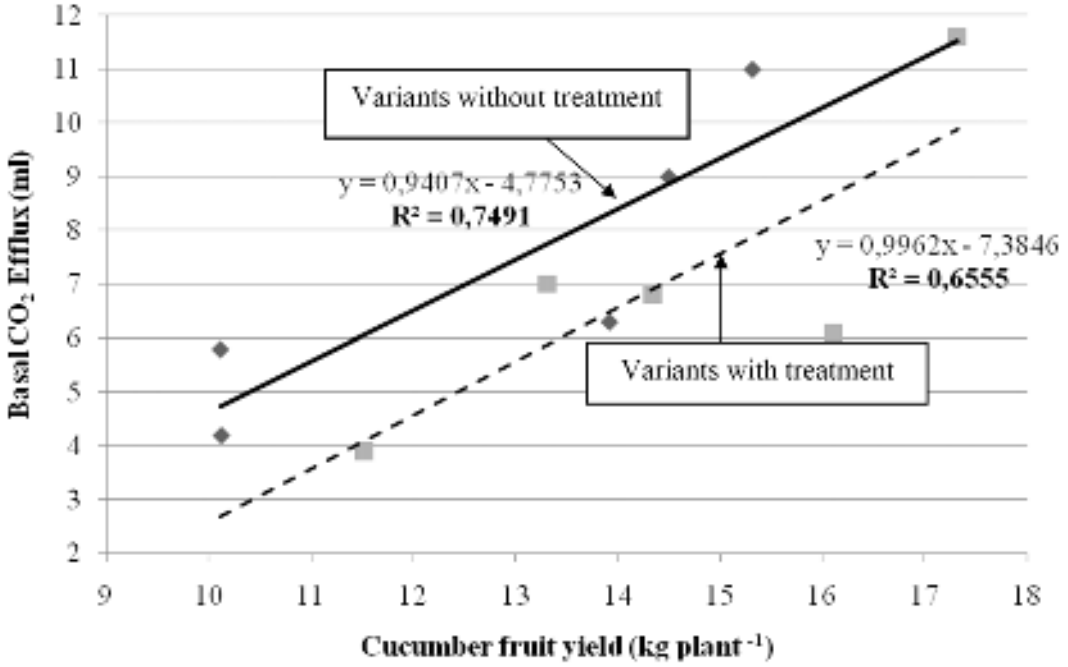


**Figure 5.52** Relation between productivity of cucumber plants and basal $CO_2$ efflux of horticultural substrates

Nitrate analysis was undertaken because some organic substrates (peat and coir) may accumulate significant amounts of nitrogen ($N\text{-}NO_3^-$) influencing thereby the microbial communities.

Results of the nitrate analysis showed that water extraction of peat and coir substrates contained the highest amounts of $N\text{-}NO_3^-$(Figure 5.54). Treated and untreated samples of peat and coir do not exhibit statistically significant difference in their nitrate values.

The highest values of $N\text{-}NO_3^-$ content of the treated peat and coir correspond with the highest integrated glucose-induced $CO_2$ efflux. These variants in the non-amended treatments there was no stimulating effect of high nitrate content on the glucose-induced respiration.

The glucose-induced respiration showed that the application of the mixture of *Bacillus subtilis* (FZB24), Lactate (LACTOFOL®) and K-Humate to the substrates contributed to higher microbial activity in the substrates expressed higher integrated $CO_2$ efflux with perlite, coir and peat. The higher respiration in the organic substrates can be attributed to the fact that peat and coir naturally can absorb and retain the significant amounts of nitrogen and other nutrients from the nutrient solution.

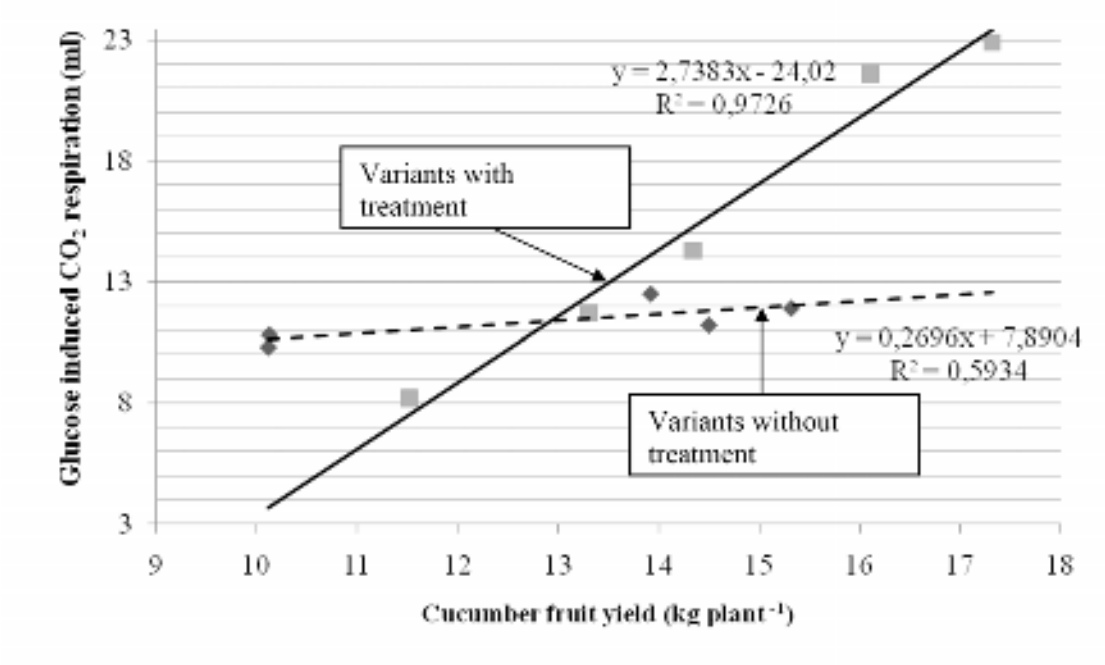


**Figure 5.53** Relation between glucose induced respiration of the substrates and plants productivity

The highest cucumber yield was achieved in the peat and coir, and the lowest in rockwool and sheep wool (Table 5.54). In all the substrates treated with the biostimulator mixture, the yield was higher in comparison to the untreated substrates. It can be assumed that application of the biostimulator mixture improves plant development in particular the root system.

Apart from these effects it seems substrates like peat and coir accumulate substantial amounts of nutrient elements, especially nitrogen. Thereby, there are surplus nitrogen for pant growth and development of microorganisms. Evaluation of the $CO_2$ efflux in the substrates and the yield of cucumbers showed in particular in variants with biostimulator addition very high correlation ($r^2$=0.97) (Figure 5.53). Different basal and glucose-induced respirations are attributed to differences in substrate nature. Different physical properties of substrates lead to either to leaching or accumulation of nutrient elements. Nitrogen is an element that is used in metabolic processes of both microorganisms and plants. This fact brings up an assumption that biological development and productivity of the plants in horticultural production may depend on both nutrient availability and status of the microbial community. Figure 5.54 illustrates this assumption. The variants with peat and coir that gave maximum result in terms of productivity of the cucumber plants have also accumulated the highest concentration of N-$NO_3$. $CO_2$ efflux as a function of the microbiological activity of the substrates describes the status of the microbial community at the final stage of the vegetation experiment.

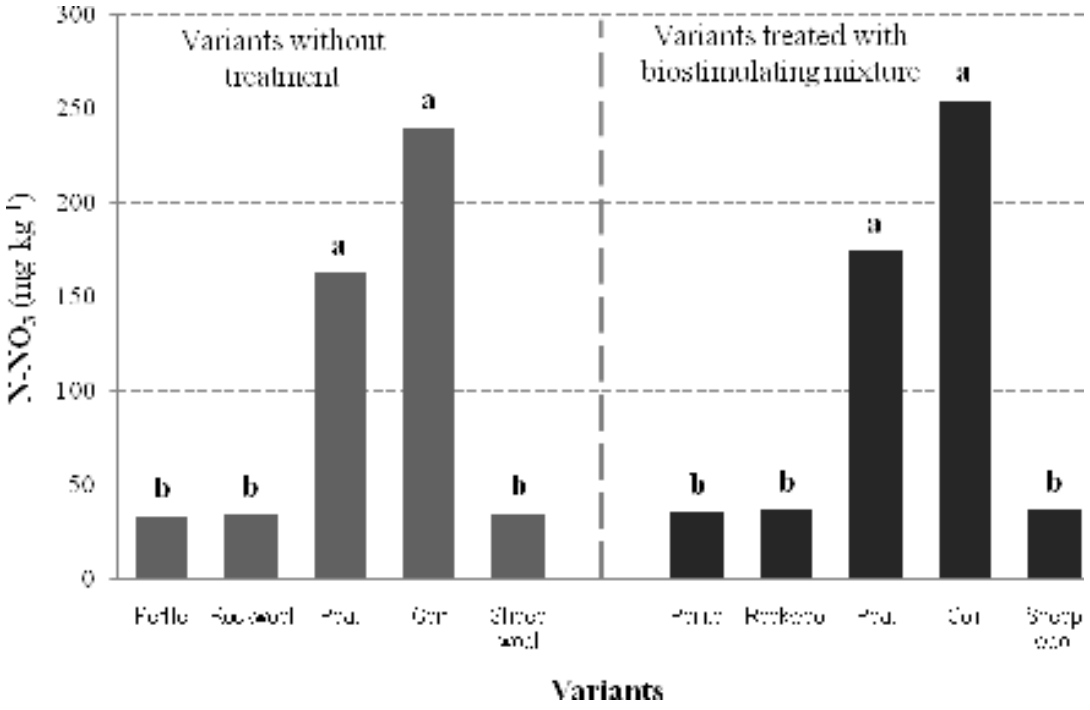


**Figure 5.54** Nitrates content in the substrates. Two-way ANOVA Tucky's p=0.05. Different letters indicate statistically significant differences. n= 30

The increased $CO_2$ respiration is incident to organic substrates of both treated and non-treated variants. Correlation between plant's productivity and glucose-induced $CO_2$ efflux attests influence of microbial community of substrates on yield formation.

**Discussion**

Microbiological status of the horticultural substrates represents an important research issue. This importance is derived from the fact that cultivation of cucumber plants in this research takes place in the different substrates and that means different conditions for rhizospheric conditions. Application of the biostimulating mixture for stabilizing plant system during the vegetation period include a bacteria – *Bacillus subtilis* FZB 24 which is used due to its multipronged beneficial effects in creation optimal microbiological conditions in the root system. The five different substrates – perlite, rockwool, peat, coir and sheep wool were in use during two-year vegetation experiment. In the course of this experiment four fruit rotations were conducted. Each fruit rotations had treated variants – with application of biostimulating mixture (Lactofol "O"+ K-Humate + *B.subtilis* FZB24) and without treatments. After last rotation when all cucumber plants were removed and substrate samples were taken to evaluate respiration of substrates after

four rotations. Use of SIR-Method – generally applied with soil samples, allowed evaluation the activity of microbial community in the horticultural substrates.

This experiment showed that conducted treatments resulted in change of microbiological activity of horticultural substrates. This change can be seen on the organic substrates (peat and coir). The variants with peat and coir that gave maximum result in terms of productivity of cucumber plants have also accumulated highest concentration of $N\text{-}NO_3$ and this can be a key factor for creating a viable microbial community. On the other hand application of lactate in form of Lactofol "O" can serve as an additional substrate for microorganisms at leas on the initial stages of the experiment.

# 6 General discussion

If a plant in hydroponical systems has a suboptimal growth condition – as a function, for instance, of micronutrient deficiency in that particular substrate, it becomes weakened and easy prey for diseases, and once these diseases established themselves on one plant, they are readily available for other plants and that is only a question of time when they take on them. I think in this instance we can think of this one discrete plant` nutritional status as a variable that can be either transferred on other plants or influenced through mechanisms known to us. In this sense plant, speaking mathematically is not a material point that can be neglected but a vector which can be characterized in terms of direction, and in this case this vector points at specific developmental stage of the plants in the future. Moreover this vector and thus its direction it points to, can be influenced by employing mechanisms we already aware of. The question - what are these mechanisms? To answer this, we can consider the reaction patterns of plants on certain changes in their environment. Plants can react on changes in their environment on very early stages. Changes in air temperature by one degree centigrade, already finds its reflection in plants metabolism. Plants interact with substrates, other plants, microbial community, inorganic world (nutrients) on permanent basis. Any sort of interaction in biological or even mechanical system has a precondition – namely - interface. For plants it's a surface of their roots, blades of their leaves, etc. How does such an interaction occur? The answer is - signal molecules. Plants and microorganisms operate through common interaction facet, in the way where they employ signal molecules as a mean of communication by which transfer of mass, information and energy occur. That is exactly where we can regulate the situation by introduction of the biostimulating agents or plant strengtheners of interest.

**Humates**

Two types of humates were used in the current study: Fe-Humate and K-Humate. Both these substances proved to have positive influence on the development of cucumber plants and formation of cucumber fruit yield. The influence of these humates on the cucumber plants was different and it can be explained by the fact that Fe-humate – substances that contains at least 6% (in this research 8%) of iron can be used to reduce iron deficiency during vegetation. Fe-Humate derived from two different types of raw material the Russian (R) and the German (G) leonardite. K-Humate, however, is a substance that is primarily designed for soil amendment. That means that this substance can be used primarily on horticultural substrates for improvement of their physical properties (CEC, pH). This also can explain the negative effects of the foliar application

of K-Humate. Fe-humate, on the other hand proved to be more effective in leaf treatment in comparison to foliar application of K-Humate. However, their influence on cucumber plants was different under comparable conditions. For instance, application of HUMIRON Fe 8% (R) in concentration of 0.001% proved to increase the leaf quantity on the cucumber plants to 25 leaves on the variant with standard nutrient solution and to 18 on the variant with iron deficiency.

Since iron deficiency was modulated from very start of experiment and treatments with Fe-humate were conducted later in the course of vegetation and only once, an inference can be made that application of iron-humate to alleviate deficiency of iron was not entirely successful. At the same time, application of HUMIRON Fe 8% (G) in the same concentration showed significantly different result of 28 leaves, whilst on the variant with standard nutrient solution and 25 leaves with nutrient solution without iron. Obviously, the availability of iron in the nutrient solution did play limiting role in the development of the cucumber plants, but at the same time, it is very rare that such a situation can occur during the vegetation.

Iron deficiency, as limiting factor in cucumber plant development, reflected itself in formation of assimilation apparatus of the plants. The leaf area on the variants with and without iron-deficiency in nutrient solution under other comparable conditions was 3945 $cm^2$ and 4012 $cm^2$ respectively. In the experiments it was shown that gradual increase of concentration of Fe-humates of both types reflects negatively on developmental patterns of cucumber plants. Formation of the fruit yield of the cucumber plants depends on availability of iron in the nutrient solution. At the same time, application of Fe-humate on the variants with iron-deficient nutrient solution did not influence yield increase of cucumbers. 10-fold increase in concentration of HUMIRON Fe 8% (G) to 0.2% showed decrease in leaf area – 3457$cm^2$. Developed leaf number that was achieved on the variant with application of 0.1% of Fe-humate (G) one can see that concentrations of iron-humates of different raw inhibited formation of larger amount of the cucumber fruit. Nevertheless, it was established that, deficiency of iron in nutrient solution is a factor that decreases productivity of cucumber plants which is seen on control variants of both with and without iron nutrient solution. The yield of cucumber fruits shows the same pattern as fruit number. Several variants in experiment with Fe-deficiency show statistically significant increase in fresh matter content of leaves. Only in one case of HUMIRON Fe 8% (R) 0.001% there is increase in fresh matter of the stems. Increase in concentration of HUMIRON Fe 8% (R) brought statistically significant increment in fresh matter of cucumber plants whilst application of HUMIRON Fe 8% (G) contributed to decrement of the fresh matter. Plant cultivation in hydroponical systems is quite problematic concerning the proper balancing of the nutrient

supply. According to previous investigations, humates have a favourable effect on the nutrient supply of horticultural crops. Therefore the application of humates was tested as an approach to improve both the nutrient balance and plant vitality. According to Tattini *et al.*, (1990) and Adani *et al.,* (1998) humic acid promotes the uptake of N, P, Fe and Cu of tomato and other plants. The positive effect of humic acid on the uptake of N, P, Fe and Zn was also proved with corn plants (FORTUN and LOPEZ, 1982). Moreover, humates influence the respiration-process, the amount of sugars, amino acids and nitrate accumulated, and make the plants resistant against diseases and viruses. Delivery of the nutrient elements can be achieved either through balanced composition of nutrient solution from the start of the vegetation or through nutritional supplements in critical phases of the plant's growth. Example of the iron deficiency that was brought up in the first experiment shows that although the iron humate can be used to relieve the short term deficiency of iron, it cannot be used on the permanent basis. The iron-humate is delivered to plants by the watering system but at the risk of the pipe clogging; otherwise it is extremely work intensive process that in practice can render entire greenhouse production unprofitable. The balanced nutrient solution is always preferable to one-off supplements.

The aim of theses experiments was to investigate the effect of application of a well soluble Fe-humate (HUMIRON®) on rhizosphere and leaves, respectively on the growth and yield of cucumbers. It is assumed that humic acids have special importance for transportation and availability of microelements in the plants (DAVID *et al.,* 1994). Chlorosis could be prevented, by humate application; probably because the availability of iron was enhanced (FORTUN and LOPEZ, 1982; ALVAREZ *et al.,* 1996; KREIJ and HOEVEN, 1997).

Existing different types of humates are being used to regulate plants growth and development, especially on critical stages of development. Different applications of $NH_4$-, K- or Ca-humates are being used and positive effects on plant growth are proved (Hoang, 2003). The background of these effects, however, is not completely clarified. But it can be assumed that the effects are not explicable with the content of nutrient applied together with the humates because their concentration is very low. Now, humates with a high content of metal-ions are available. This amount of metal-ions bound on humate could influence the content of micronutrients in the nutrient solutions directly. Being able to improve the uptake of the nutrient elements by the plants, humates contribute to enhanced root development and the root activity (exudates) which in turn improves microbiological activity in the substrate and particular – in the root zone (MALCOLM and VAUGHAN, 1979).

***Bacillus subtilis* FZB 24**

Supporting development of benign microorganisms in the hydroponical system may induce higher quality of the produce. Interaction between microbial community substrate and horticultural plants contributes to protection against diseases. Application of microorganisms as biological control agents and plant growth-promoting rhizobacteria proved to be effective in improving plants productivity of the cucumber plants. The results of this research confirm positive effects in utilization of *B.subtilis* FZB 24 as plant strengthener that contributes to higher plants productivity. On the other hand, horticultural practices can be confronted by different suboptimal conditions that, as reported by Stragier and Losick (1996) can lead to reduced activity of *B.subtilis*. In fact *B.subtilis* in response to the nutrient starvation develops endospores. In such a state it resists a variety of harsh external conditions, such as extreme cold or heat. Hiltner in 1904 first described what he called "the rhizosphere effect," where he assumes that many microorganisms are attracted to nutrients exuded by plant roots. FARRAR J.F, JONES D.L. (2000) describe activation of microbial activity through stimulation by the release of soluble sugars from the roots but at the same time, organic anion exudates can support microbial growth as well (McKEEN *et al.,* 1986). The microorganisms themselves are in position to secrete molecule compounds into their environment (PRIEST *et al*., 1977). Bacteria also play a very important role in degrading plant- and microbe-produced compounds in the soil that would otherwise be allelopathic or even toxic to next generations of microorganisms as well as higher plants (HOLDEN *et al.*, 1999). The bacteria such as *B.subtilis*, can also positively interact with plants by producing protective biofilms or antibiotics operating as biocontrol against potential pathogens that contributes to formation of positive microbial community within root area of the plant (BAIS *et al.,* 2004.), but for that to happen - root colonization is important as the first step in infection by soil-borne pathogens and beneficial associations with microorganisms (GROSCH *et al.,* 1999).

**Lactofol "O"**

Lactates, salts of lactic acid, can be also used to chelate nutrients, especially micronutrients. The suspension fertilizer consists of a biotechnological product (liquid phase) and a solid phase comprising nutrient macro- and micro-elements.

LACTOFOL"O" – salt of lactic acid saturated with micro- and macronutrients, is designed as foliar fertilizer. The mechanism of uptake and transport of foliar applied nutrients involves a complex plant tissue system including dermal, vascular, and ground systems (Rathore, 2000).

LACTOFOL"O" as foliar fertilizer - increases leaf area of the plants in comparison to control variants this results are confirmed by the findings of other scientists (Römheldl *et al*., 1999). As it was shown in this research, lactate increases elongation of the root and stem of the cucumber plants. Application of lactate as root fertilizer showed the same effects as in the variants with the foliar treatments.

Stress reducing effects of lactates could be found especially in combination with the nutrient solutions with too low or too high pH values. The same effects can also be found under the suboptimal growing conditions, for example, with extreme air temperature fluctuations (BOEHME *et al.,* 2000). Lactofol® is a suspension fertilizer for leaf nutrition of plants. The suspension fertilizer "Lactofol" was developed for leaf nutrition of agricultural plants. Its use results in an average 8 to 10% increase in the yields of wheat, and in an increase in protein content of the grains of 0.5 to 1%.

Lactic acid is known to be strong chelating agent and is capable to bind variety of nutrient elements in form of cations in its coordination area and indeed Rankov (1992) confirms this effects. In purely chemical terms, the reversibility of chelating bounds makes it very useful in leaf application and allows to support plants with nutrient elements in critical staged of their development (SAPUNDJIEVA *et al.,* 1997). Upon application of Lactofol® on leaf surface, chelated cations of Lactate travel against concentration gradient from its coordination area into leaf apparatus and enter metabolism processes. The leaf application of Lactofol is helpful in dealing with micronutrient deficiency during vegetation period of horticultural plants (KERIN, 1997). LACTOFOL"O" contains ions of metals like $Fe^{3+}$, $Zn^{2+}$, $Mn^{2+}$, $Co^{2+}$ positively influence photosynthetic activity of plants (STOEVA and SHABAN, 2002). At the same time in our experiments we were confronted with a negative side of the leaf application of the lactates. Uneven development of the air temperature and relative air humidity can lead to the outbreak of the mildew of the cucumber plants. On the other hand, this problem can be solved by using the mildew-resistant cultivars of the horticultural crops.

Lactofol"O"® - is a suspension of micro- and macro- elements in concentrated form. It contains nitrogen in form of ammonium ($NH_4$), nitrate ($NO_3^-$) and amide (SAPUNDJIEVA *et al.,* 1997). Upon application of Lactofol these forms of nitrogen enter and increase metabolism in the plant (RANKOV, 1992). Increase of metabolism can be expected due to availability of $NH_4$ in the lactate. Incorporation of nitrogen in the form of ammonium takes much less time whilst it is in reduced form in comparison to nitrate (SAPUNDJIEVA *et al.,* 1997). The problem here is that absorbed nitrogen is useless unless it is incorporated into organic molecules. Incorporation of

nitrogen, especially in the form of nitrate, generally occurs in the root system of plants and requires availability of carbohydrates – products of photosynthesis. Thus application of LACTOFOL"O" as leaf fertilizer can lead to accumulation of nitrates in plant's tissues and potentially reduce quality parameters of end product.

Creation of optimal nutritional conditions in the substrate is a very important task from the onset of the plants growth (RANKOV, 1992). Utilization of chelating compounds helped to balance availability of micronutrients for the plant root system. The possible solution for this problem is an application of different substances of organic nature. Different by the structure, these substrates are capable of retaining macro and microelements, making them available for root systems. Many present conceptions lactate application are focused on the use of the Lactofol "O" – the first formulation. At present, there are many formulations based on the biological needs of the particular plants. The treatment of plants with specific formulations of the lactate can address the plant's needs, especially, in micronutrients.

**Biostimulating mixture**

Different growth conditions can lead to the manifold development problems for the horticultural plants. We have already seen that the application of the certain biostimulating substances can relieve the plants from the lasting effects of the suboptimal growth factors. Because of the complexity of interaction between the plant and the environment, the application of only one substance can represent a barrier to solving the stress relieving problems.

The idea of biostimulating mixture – mixture of lactates humates and microorganisms proved to have beneficial effects in hydroponics of the gram-negative rhizobacterium *Bacillus subtilis* FZB 24® regarding the reduction of salt stress (BOEHME, 1999) and its effects against fungal and bacterial diseases are also proved (LOEFFLER *et al.,* 1986; KREBS *et al.,* 1998; SCHMIEDEKNECHT *et al.,* 1998; GROSCH *et al.,* 1999).

Beside microorganisms, also organic substances with different chemical composition can be used as biostimulators, e.g. humates and lactates. Also for these substances growth stimulating and stress-reducing effects could be shown in hydroponics (BOEHME, 1999; BOEHME *et al.,* 2000; HOANG, 2003). Humates well know, as main components of soil fertility have so far more or less no importance in hydroponics. However, some very interesting effects of humates are described concerning their stimulating effect on nutrient uptake (FORTUN und LOPEZ, 1982; TATTINI *et al.,* 1989), counteracting salt and drought stress as well as temperature stress. The positive effect of humates on availability and uptake of nutrients like calcium, magnesium,

and phosphorus due to chelating as well as their combinations with microorganisms and lactates widens activity spectrum of such a mixture in comparison to their separate application.

From current research it can be concluded that application of lactates in form of Lactofol "O" increases the number of marketable fruits in comparison with the control as well as variants treated with biostimulators. Although developed as leaf fertilizer, LACTOFOL"O" proved to increase productivity of plants by its application in the root area of the plants. After treatment of roots with LACTOFOL"O" and *Bacillus subtilis* and K-Humate should be taken into consideration because these additional applications could enhance the yield further. This could be especially important in long time cultivation. The treatments affected the photosynthetic conditions of the plants and the plant especially the root growth in pH and temperature stress situation. Another example from this work is a temperature stress**.** Temperature stress was applied by decreasing air temperature in climate chamber right after third treatment by biostimulating mixture. The effect of the biostimulator mixture leads to a significant difference in all parameters in comparison with the non treated variant, except for the leaf area. It can be assumed that plants with a well developed root system have higher resistance against different stress situations. The biostimulator mixture used in these experiments had also in previous experiments a positive reaction on the root growth (BÖHME, 1999). On the other hand in the experiment with treatments of the biostimulator very close relationship ($R^2$ linear = 0.940) between green biomass and mass of roots was found. This close relationship confirms the hypothesis, that increase in root mass leads to formation of larger shoot and leaf mass of test plants even if those plants were influenced by stress conditions if such biostimulator is treated.

**Microbiological activity of the substrates**

Investigation microbiological activity of horticultural substrate was confronted with a problem of method choice. Probably the first experiments with were conducted and described by Carlile et al., (1991) where he used glucose for triggering microbial response. A disadvantage of his approach can be derived from the fact that in these experiments he did not elaborate on his choice of glucose concentration, which is certainly not a case in this study. 2, 3 and 4 mg glucose $gram^{-1}$ substrate were specially tested to choose the most appropriate response of microbes in the substrate. These concentrations were tested with perlite, rockwool, coir, peat and sheep wool. It was established that 4 mg $gramm^{-1}$ substrate is the most appropriate concentration for testing microbiological response of the substrates. It can be assumed however, that further increase in this concentration can be deemed appropriate.

Glucose-induced respiration in our experiments showed that long term treatment (four fruit rotations – 12 treatments) by mixture of *Bacillus subtilis* (FZB24), lactate (LACTOFOL®) and K-Humate contributed to the creation of more active microbial community in the substrates what finds its expression in higher integrated $CO_2$ efflux on perlite, coir and peat. Higher respiration in organic substrates can be attributed to the fact that peat and coir naturally can absorb and retain significant amounts of nitrogen and other nutrients from nutrient solution. The method employed here can be used for evaluation of horticultural substrates and has a benefit of measuring $CO_2$ efflux of microbial biomass incident to given substrate without interference of atmospheric conditions which is a fact in direct measurements usually conducted in greenhouses.

**Substrates**

Application of combined biostimulating mixture (0.1% K-Humate, 0.2% LACTOFOL"O", spore suspension 0.2% ($10^7$ cfu/ml) of *Bacillus subtilis* FZB 24®) leads to improved plant development that include formation of extensive root system. These processes lead to gradual increase in bulk density of substrates and decrease in water holding capacity. Substrates like peat and coir accumulate substantial amounts of nutrient elements, especially nitrogen. These variants demonstrate best productivity especially in third and fourth vegetations. The substrates used distinguish concerning their physical properties. Extreme differences regarding the air and water capacity could be estimated before the experiments were started. Sheep wool exhibited with about 70% the highest air capacity while peat had the lowest air capacities. In contrast the water capacity of sheep wool was very low due to the properties of the wool.

## Conclusions

The plant system is a complex one and thus requires multipronged approach in regulating its efficiency and sustainability. Application of biostimulating substances of different nature offers such possibility. Combination of microorganisms lactates and humates was most effective in assuring sustainable plant's productivity during long term cultivation. Several substances were tested. Among those are K-Humate HUMIRON Fe ®; Lactofol "O"; *B. subtilis FZB* 24®. Their application on cucumber plants brought following conclusions. HUMIRON® can be used to improve plant growth and yield in substrate culture of cucumber.

It is possible to apply HUMIRON® in the rhizosphere and on leaves as well. The influence of humate shows statistically significant effects in experiments with Fe-deficiency and with

standard nutrient solution. The effect was dependent on the concentration used and 0.2% HUMIRON® was inhibiting for yield.

Application of HUMIRON Fe ®; Lactofol "O"; *B. subtilis FZB* 24® and its mixture as combined biostimulating substance had different effects on productivity and growth of cucumber plants. Root treatments proved to be most effective in forming statistically significant maximum yield of cucumber fruits. Analysis of photosystem II showed that variants with leaf treatment display values of Fv/Fm significantly lower of optimal value 0,78. Variants with root treatment exhibit higher electron efficiency of photosystem II and thus have greater potential for forming assimilation apparatus which in turn positively reflects on plant's productivity.

Experiments with different leaf treatments are only possible in combination with cultivars resistant to mildew. In all experiments where leaf treatments were undertaken, the problem of mildew arose. At the same time, treatments proved ineffective in stimulating productivity of plants. Humate treatments did brought comparable results of plants' growth and productivity but at the same time it fell short of expected standards that are common in industrial practices where productivity of cucumber plants should not be less 15 kg*plant$^{-1}$. It brings an assumption that leaf application of humates alone cannot be used in stabilizing functionality of plant's system.

**Biostimulating mixture and abiotic stresses**

Application of the biostimulating mixture containing *Bacillus subtilis* FZB24 (0.2%), K-Humate (0.01%) and LACTOFOL"O" (0.1%) proved to be useful in reducing stress influence on the growth of cucumber plants. The pH- and the temperature stress reduced the growth parameters of the plants much higher if no biostimulator was treated. A very effective and potent non-destructive method has been introduced into practice during the last decade, the chlorophyll-*a* fluorescence. Measurement of chlorophyll-*a* fluorescence is a helpful tool for determination of plant stress at early stages (KRAUSE and WEIS, 1984). In physiological terms chlorophyll-*a* fluorescence of photosystem II is an indicator for occurring photosynthetic processes (KRAUSE and WEIS, 1991; EDWARDS and BAKER, 1993). Dark adaptation precedes measurement of chlorophyll *a* fluorescence due to the fact that all photochemical and non-photochemical processes under this circumstance are relaxed. If dark adaptation is followed by illumination we can expect changes in Fo of dark-adapted yield of chlorophyll fluorescence. Following illumination with a brief pulse of extremely bright light (>4000 μmol m$^{-2}$ s$^{-1}$) saturates electron transport through PSII and gives us an opportunity to measure Fm, the maximum yield of chlorophyll fluorescence. The Fv/Fm (Yield) is to be considered as most important parameter

that can be enquired in the process of measurement taking of dark adapted sample (leaf). For a healthy plant this will typically range between 0.80 and 0.83. Lower values indicate damage to photosystem II. As it was shown in experiments with suboptimal abiotic factors like temperature and pH-stress, values of Fv/Fm (Yield) can fall as low as 0.630 as a reaction on chilling stress.

The chlorophyll fluorescence Fv/Fm-value showed the positive effect of the curative biostimulator treatments for stress counteraction in plants. Cucumber plants with no treatment of biostimulator showed very low Fv/Fm values implying that they were not able to recover from pH- as well as temperature-stress. Results show that there is strong correlation between green biomass of treated cucumber plants and their root mass. It can be assumed that the effect of stress prevention of the used biostimulator based mainly on enhancing the root growth. This combination of biostimulator compounds in the investigated mixture can be recommended.

Application of combined biostimulating mixture (K-Humate 0.01% + Lactofol ”O” 0.1% + *B. subtilis* FZB 24® 0.2%) increases stress resistance of test plants. the electron efficiency of the photosystem II expressed through quantum yield (Fv/Fm) does not manifest any significant differences before suboptimal growth conditions pH and temperature alike but significantly higher in treated variants after the stress phase in comparison to those untreated. The stress protective properties of the biostimulating mixture related to formation of the plants root system. The correlation between root mass and biomass of cucumbers in the experiment with pH and temperature stress influences with biostimulator treatments is very strong $r^2$=0,940. It can be assumed that having more extensive root system due to treatments, plants are capable of taking up more nutrient elements delivered with nutrient solution and lactate. Lactate with its pool of micronutrients can play decisive role in supporting functionality of the photosystem II.

The effects induced by Lactofol “O” 0.01%, *B.subtilis* FZB24 0.2% and K-Humate 0.01% in different treatments resulted in different growth patterns of cucumber plants. Root application of biostimulating substances proved to be more effective in increasing dry matter content of leaves and shoots and at the same time did not have any statistically significant difference on dry matter content of fruits.

Application of combined biostimulating mixture significantly reduced number of non-marketable fruits. Variants with root treatment showed significantly lower fraction of c-class cucumbers in comparison to variants with leaf treatments except the leaf treatment variant with combined biostimulating substance. The leaf treatments contributed to development of mildew on cucumber plants that resulted in very short vegetation time and low productivity.

The long term use of horticultural substrates (perlite, rockwool, coir, peat, and sheep wool) finds its manifestation in their physical properties. Application of the combined biostimulating mixture (0.1% K-Humate, 0.2% Lactofol"O", spore suspension 0.2% ($10^7$ cfu $ml^{-1}$) of *Bacillus subtilis* FZB 24®) leads to improvement in the development of the plants growth. That includes formation of the extensive root system. These processes lead to gradual increase in bulk density of substrates and decrease in water holding capacity. Substrates like peat and coir accumulate substantial amounts of nutrient elements, especially nitrogen. These variants demonstrate best productivity especially in third and forth vegetations. The physical parameters of unused sheep wool are not appropriate following the target values except the pore volume (GEISSLER et al., 1991). After the second vegetation, however, the air and water capacity approached the target values. The best physical properties in both vegetation times were determined for peat slabs except the air capacity before planting. Nevertheless the physical properties of both substrates were quite different the yield could be similar. Maybe for such a thin layer as the sheep wool substrate the physical properties are not so important. In any case more investigations are needed concerning time course of changing of physical properties and other parameters like oxygen supply. Schröder (1994) came to conclusion the advantage of thin layer substrate is the continuous oxygen enrichment.

Sheep wool could be used for two vegetation periods. After the second use the Sheep wool was completely bounded with roots and the height of the slab was only some millimeters. Therefore a use longer the one year seems to be not recommendable.

For longer use in substrate culture perlite seems to be suitable because the high stability of the air capacity. In this stage of the experiments it is not possibly to give clear recommendation concerning the irrigation frequency and calculation of nutrient solution, more experiments are needed. In both vegetation period the application of the biostimulators resulted in higher yields on any substrate investigated and can be recommended. Nevertheless, the low water capacity of new sheep wool slabs and the probably low ion exchange capacity the high yields on this substrate encourage us to continue with these investigations.

$CO_2$ efflux as a function of microbiological activity of substrates describes status of microbial community at the final stage of vegetation experiment. Increased $CO_2$ respiration is incident to organic substrates of both treated and non-treated variants. Correlation between plant's productivity and glucose-induced $CO_2$ efflux attests influence of microbial community of substrates on yield formation.

Application of combined biostimulating mixture has statically significant positive effect on formation of green biomass of roots and shoots of cucumber plants. Fresh matter of roots and shoots of cucumber plants from variant with application of combined biostimulating substance (*B.subtilis* FZB 24 + Lactofol "O" + K-Humate) were significantly different from other variants. Application of binary combinations (B.subtilis FZB 24 +K-Humate; B.subtilis FZB 24 + Lactofol "O") had no statistically significant effects on shoots' weight. Plants from variant without application of biostimulating mixture (Control) did not show statistically significant difference in root fresh weight with variant B.subtilis FZB 24 +K-Humate. *B.subtilis* FZB 24 + Lactofol "O" + K-Humate increase productivity of cucumber plants.

The results of this research can be summarized in a following way:

1. Biostimulating mixture that consists of *B.subtilis* FZB 24, Lactofol "O",K-Humate is most effective combination of all tested in this research.
2. Biostimulating mixture increases plant productivity and assures it in the long run through improvement of plants growth, formation of extensive root system and creation of assimilation apparatus of the plants.
3. Combination of *B.subtilis* FZB 24, Lactofol "O", K-Humate has stress protective properties that are, however, noticeable only under suboptimal growing conditions.
4. Biostimulating mixture effects formation of microbial cynosis in horticultural substrates and positively influences plant development.
5. Combination of biostimulating mixture and organic substrates (peat, coir) gave best results in terms of plants productivity and can be recommended for application in large scale horticultural practices.

The scientific investigation of the biostimulating effects showed the complexity of the interactions between plant, substrate and biostimulators. The influence of the biostimulating mixture had positive effects on the test plants. At the same time, there is always a possibility to broaden field of study. In this relation there are questions that can be set aside for further scientific evaluation.

For the future experimentation it can be taken into account that application of the biostimulating substances can be optimized. This optimization concerns primarily the timing of the biostimulating mixture application, i.e. its application on different developmental stages of the horticultural plants can have positive results on yield formation.

Combination of potassium humate, Lactofol and *B.subtilis* can influence formation of the substrate microbial community. Further investigations can be done concerning evaluation of microbial activity in the rhizosphere of the plants. Utilization of the SIR-method in future

experiments will facilitate acquisition of additional data about changes in microbial activity in different horticultural substrates and under different horticultural crops.

The physiological effects resulted from application of the biostimulating mixture on the cucumber plants were focused primarily on the study of electron efficiency of Photosystem II. The future studies may be concentrated on other aspects of plant physiology. Reaction of the treated plants on the suboptimal growing conditions in comparison to the variants without treatment with biostimulating mixture may bring new knowledge of the biochemical underpinning of the stress physiology.

## Summary

Application of humates, lactates and microorganisms in agriculture takes on ever-increasing scale. Availability of biostimulating substances in form of commercial products simplifies their utilization in horticultural practice and yet implies many other questions. The major one is whether application of these compounds can contribute to sustainable practice of agricultural production. More importantly, if previous practices in dealing with humates and microorganisms report good results, can it be possible to combine some of those compounds to increase their functional capacity as a biostimulating mixture?

In this study, in the line with previewsly stated objective: to investigate of an influence of humates, lactates and *B.subtilis* FZB24 on growth and yield of *Cucumis sativus L.*, we have undertaken an attempt to define critical issues related to singular application of humates, lactate (LACTOFOL"O") and *Bacillus subtilis* FZB24 as well as their combinations with intent to broaden the spectrum of their activity within the horticultural system.

The results of this study show that combination of K-Humate, LACTOFOL"O" and *Bacillus subtilis* FZB24 possesses a string of beneficial features in comparison to their singular application. Application of the biostimulating mixture to the root system of the plant (*Cucumis sativus L*) at the beginning of the vegetation, proved to be capable to mitigate the negative effects of the short-term suboptimal growing conditions. Examination of the electron efficiency of the photosystem II of cucumber plants being exposed to chilling stress, it was established that only the plants treated with biostimulating mixture in formulation K-Humate 0.01% + LACTOFOL"O" 0.1% + *B.subtilis* FZB24 0.2% survived and regained their photosynthetic capability. The experiments with suboptimal pH values of the nutrient solution proved the hypothesis that the biostimulating mixture has stress relieving properties.

The long term experiments (4 fruit rotations) revealed other different influences of the combined application of humates, lactate and *B.subtilis* FZB24, in particular its influence on microbial community of the horticultural substrates such as perlite, rockwool, coir, peat and sheep sheep wool. The method of substrate-induced respiration (SIR) was used for evaluation of microbial activity of horticultural substrates. Being applied primarily on the soils, this methods was particular useful with horticultural substrates, although some modification were needed.The optimal amount of glucose was determined with 4 mg of glucose $g^{-1}$ of substrate.

A special integral calculation of $CO_2$ efflux over period of time was deduced and employed to evaluate differences in respiration between treatments. It can be assumed the SIR-method combined with integral calculation provide additional information on the activity of microbial community in the substrates.

Different other effects of the biostimulating mixture were analyzed. In particular, application of combined biostimulating mixture has statically significant positive effect on formation of green biomass of roots and shoots of cucumber plants. Fresh matter of roots and shoots of cucumber plants from variant with application of combined biostimulating substance were significantly different from other variants.

As an outcome of this scientific study it was established that application of combined biostimulating mixture (K-Humate 0.01% + LACTOFOL"O" 0.1% + *B.subtilis* FZB24 0.2%) has a beneficial effect on physiological reaction of cucumber plant against singular as well as binary utilization of biostimulating compounds.

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## List of figures

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**Attachment 1**

Calculation of integral $CO_2$ efflux (treated variants)

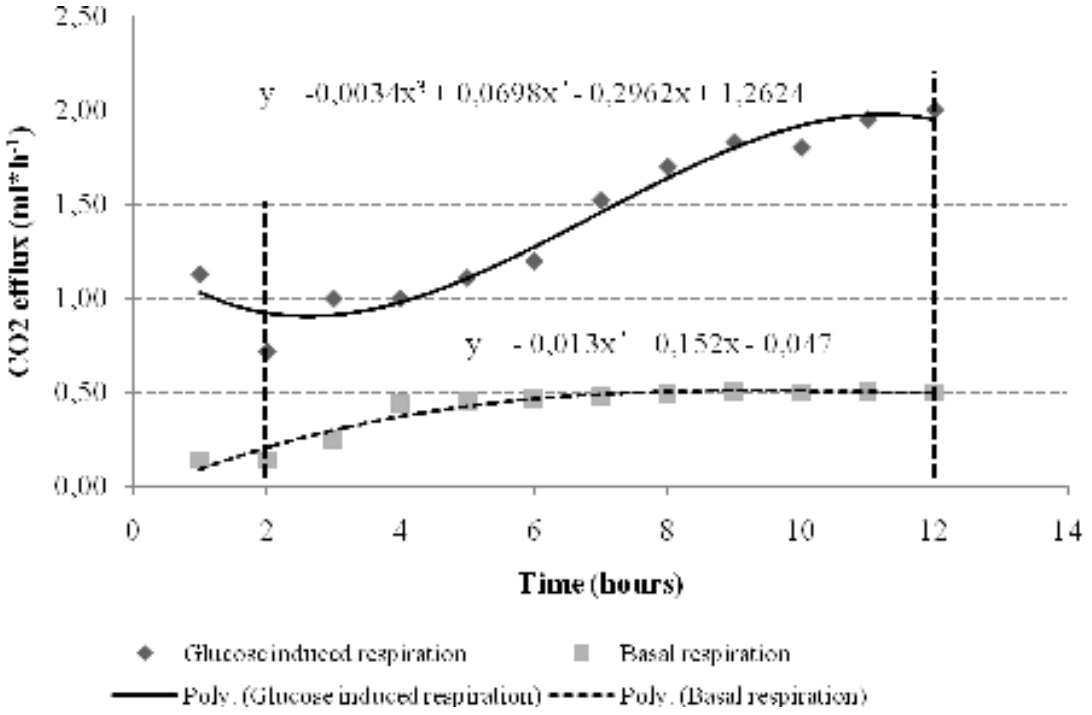


**Figure 1.** CO2 efflux on perlite with treatment (Lactofol, K-Humate, B.subtilis FZB24) (first replication)

Solution of equation for glucose induced respiration:

$$\int_2^{12} (-0.003x^3 + 0.069x^2 - 0.296x + 1.262)dx$$

$$(-0.00075x^4 + 0.023x^3 - 0.148x^2 + 1.262x)\Big|_2^{12}$$

$$-0.00075 * 12^4 + 0.023 * 12^3 - 0.148 * 12^2 + 1.262 * 12 - (-0.00075 * 2^4 + 0.023 * 2^3 - 0.148 * 2^2 + 1.262 * 2) =$$

**15.92 ml $CO_2$**

Solution of equation for basal respiration:

$$\int_2^{12} (-0.013x^2 + 0.152x + 0.047)dx$$

$$(-0.004x^3 + 0.076x^2 - 0.047x)\Big|_2^{12}$$

$$-0.004 * 12^3 + 0.076 * 12^2 - 0.047 * 12 - (-0.004 * 2^3 + 0.076 * 2^2 - 0.047 * 2) = 2.71$$

**ml $CO_2$**. ; Integral efflux: **15.92 ml $CO_2$ – 2.71 ml CO2 = 13.21 ml $CO_2$**

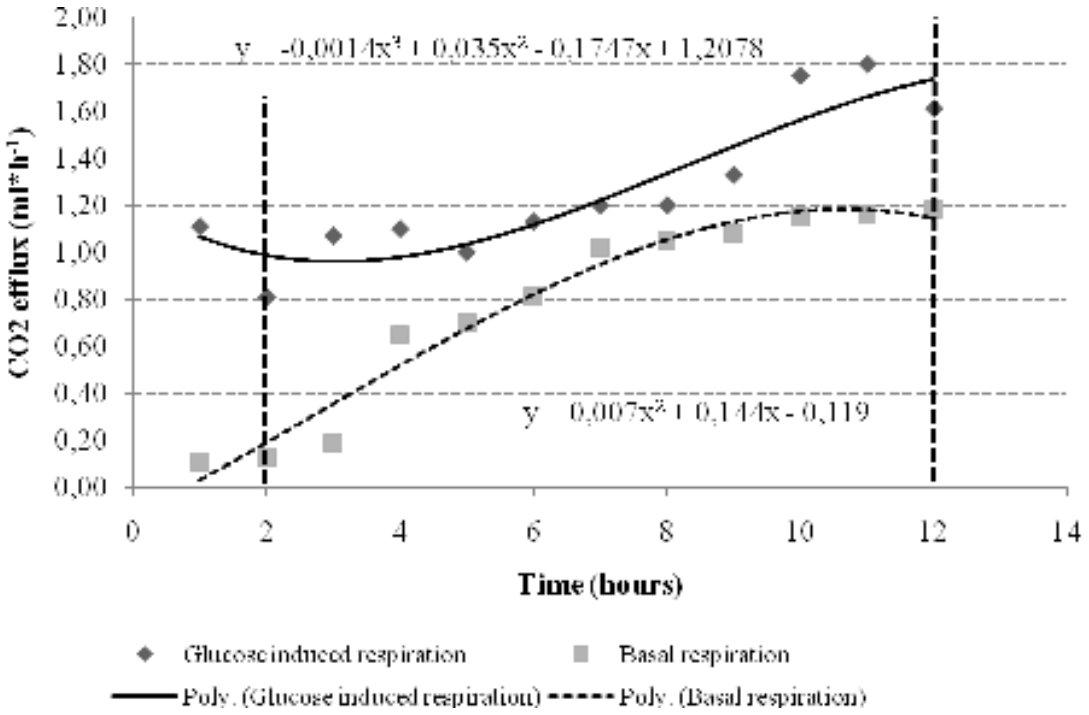


**Figure 2.** $CO_2$ efflux of on perlite with treatment (Lactofol, K-Humate, *B.subtilis FZB24*) (second replication)

Solution of equation for glucose induced respiration:

$$\int_2^{12} (-0.001x^3 + 0.035x^2 - 0.174x + 1.207)dx$$

$$(-0.00025x^4 + 0.0117x^3 - 0.087x^2 + 1.207x)\Big|_2^{12}$$

$$-0.00025 * 12^4 + 0.0117 * 12^3 - 0.087 * 12^2 + 1.207 * 12 - (-0.00025 * 2^4 + 0.0117 * 2^3 - 0.087 * 2^2 + 1.207 * 2) =$$

**14.77ml $CO_2$.**

Solution of equation for basal respiration:

$$\int_2^{12} (-0.007x^2 + 0.144x - 0.119)dx$$

$$(-0.002x^3 + 0.072x^2 - 0.119x)\Big|_2^{12}$$

$$-0.002 * 12^3 + 0.072 * 12^2 - 0.119 * 12 - (-0.002 * 2^3 + 0.072 * 2^2 - 0.119 * 2) = \textbf{12.09 ml } CO_2.$$

Integral efflux: **14.77ml $CO_2$ - 12.09ml $CO_2$ = 2.68 ml $CO_2$**

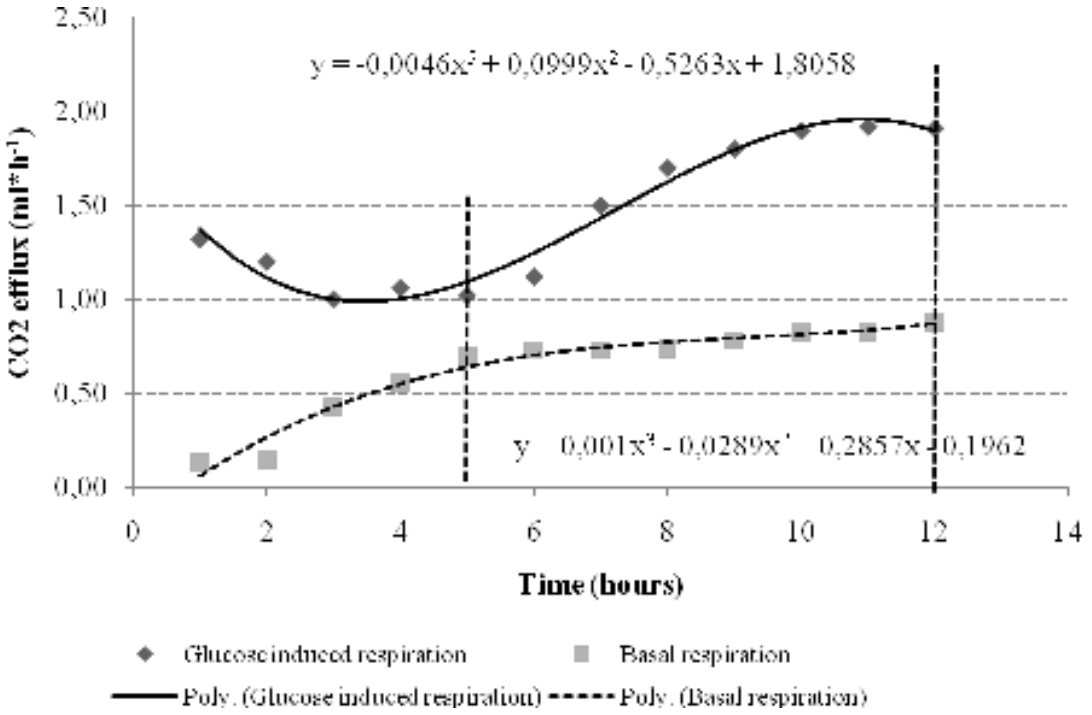


**Figure 3.** $CO_2$ efflux of on perlite with treatment (Lactofol, K-Humate, *B.subtilis FZB24*) (third replication)

Solution of equation for glucose induced respiration:

$$\int_5^{12} (-0.004x^3 + 0.099x^2 - 0.526x + 1.805)dx$$

$$(-0.001x^4 + 0.033x^3 - 0.263x^2 + 1.805x)\Big|_5^{12}$$

$$-0.001 * 12^4 + 0.033 * 12^3 - 0.263 * 12^2 + 1.805 * 12 - (-0.001 * 5^4 + 0.033 * 5^3 - 0.263 * 5^2 + 1.805 * 5) =$$

**14.13 ml $CO_2$**.

Solution of equation for basal respiration:

$$\int_5^{12} (0{,}001x3 - 0{,}028x2 + 0{,}285x - 0{,}196)dx$$

$$(-0.00025x^4 + 0.009x^3 - 0.143x^2 + 0.196x)\Big|_5^{12}$$

$$(-0.00025 * 12^4 + 0.009 * 12^3 - 0.143 * 12^2 + 0.196 * 12(-0.00025 * 5^4 + 0.009 * 5^3 - 0.143 * 5^2 + 0.196 * 5) =$$

**5.65 ml $CO_2$**.

Integral efflux: 14.13ml $CO_2$ – 5.56ml $CO_2$ = 8,57 ml $CO_2$