Influence of Endophytic Mycoflora of Xerophytes on Drought Resistance of Pepper (*Capsicum Annum* L.)

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***Abstract*—** *Because of significant increase of temperature, decrease in seasonal precipitations and hard and frequent droughts on the background of climate global change the attenuation of the yield of many agricultural crops is expected. Different approaches are used to raise plant's drought resistance. Applying plants endophytes is one of the modern ways of problem solwing. The purpose of the presented work was to study the effect of spraying the pepper plant (Capsicum annum L.) with consortia of endophytic microscopic fungi of wild xerophytes on experimental plant's drought resistance and productivity. Ten different species of xerophytes served as a source of endophytic fungi: Artemisia lerchiana Weber ex Stechm., Artemisia scoparia Waldst. &Kit., Erigeron canadensis L. (Conyza canadensis), Lactuca serriola L., Setaria viridis (L.) P. Beauv., Ballota nigra L., Polygonum aviculare L., Tribulus terrestris L., Zygophillum fabago L., and Chenopodium album L. Isolation of microscopic fungi was performed following the standard scheme, i.e. the primary sowings and later – the pure cultures were obtained. The efficiency of the fungal biopreparation was evaluated by the biometrical and biochemical indices of plants. For this purpose the habitus of both control and sptayed plants was observed and the height was measured; as well as the content of plastid pigments and ascorbic acid in leaves was studied. According to experimental results is clear that microscopic fungi alleviated the stress effect on experimental plants and enhanced the drought resistance of photosynthetic apparatus. Biopreparations stimulated the growth and development of test-plants as well, and accelerated the flowering onset.*

***Keywords*—** *Drought Resistance, Endophytic Fungi, Pepper, Xerophytes*.

I. INTRODUCTION

Significant increase of temperature, decrease in seasonal precipitations and hard and frequent droughts are expectable on the background of climate change in nearest decades. Probability of plants dying out because of hard stresses caused by such conditions is very high. Attenuation of the yield of many agricultural crops is expected as well (Rezaei et al, 2023).

Different approaches are used by scientists to raise plant's drought resistance (Yasha et al., 2017). One of the modern ways of the problem solwing is applying of plant endophytes (Kim et al., 2012).

It is well known that a big part of the plant's microbiome is occupied by endophytic fungi, which perform their life cycle inside the plant without any apparent damage to the host (Grabka et al., 2022). Applying the biopotential of endophytic fungi to increase plant drought resistance and productivity is very popular today. Many-sided and intensive investigations of the abilities of endophytic mycobiota have been done during the last peiod (Kour et al., 2019).

To study the effect of the consortium created from the endophytic microscopic fungi, isolated from wild xerophytes, on the drought resistance and productivity of pepper plant (*Capsicum annum* L.) was the purpose of the presented work.

II. MATERIALS AND METHODS

2.1 Biological material and place of collection:

Ten different species of wild drought resistant plants were collected on a dry habitat, situated at the suborb of Tbilisi (end of the Mindeli st.), as a source of endophytic fungi: *Artemisia lerchiana* Weber ex Stechm., *Artemisia scoparia* Waldst. &Kit., *Erigeron canadensis* L. (Conyza canadensis), *Lactuca serriola* L., *Setaria viridis* (L.) P. Beauv., *Ballota nigra* L., *Polygonum aviculare* L., *Tribulus terrestris* L., *Zygophillum fabago* L., and *Chenopodium album* L.

Plant samples were collected following standards, maintaining maximum sterility. Leaves and stems of plants were placed in a streile container with reference of species name and location.

2.2 Isolation of endophytic microscopic fungi from xerophytes:

Isolation of microscopic fungi was performed following the standard scheme, i.e. the primary sowings and later – the pure cultures were obtained. The samples were preliminary treated according the standard method (Vujanovic et al., 2000). Later the preliminary treated piece of the sample was placed on a Petri dish maintaining the sterility, on the the following universal agarised nutrient medium (per liter): must – 0.5lml, tap water – 0,5lml, agar – 21g; pH of the nutrient medium – 5,5-5,8; sterilization regimen – 121^{o}C, 15min. The primary sowings were obtaind by the sample dilution method as well, on the above mentioned agarized universal nutrient medium (Waksman, 1916). The primary sowings of the microscopic fungi were incubated on Petri dishes at 28^{o}-30^{o}C, in thermostat for 10days.

2.3 Obtaining pure cultures of microscopic fungi:

After the primary sowings were grown a small part of their micelium was looped on a Petri dish containing a sterile agarized nutrient medium. Microbiological inoculums were incubated in a thermostat at 25-28°C. This procedure was repeated several times, until a pure culture was obtained on a separate plate. Pure cultures were stored in test tubes, on universal agarized, slanted nutrient medium, in a refrigerator at 4°C.

2.4 Identification of the microscopic fungi isolated from drought resistant plants:

The primary identification of microscopic fungi was based on macromorphological and microscopic research methods, comprising visual characterization of the cultural-morphological properties (growth rate, diameter, size, color, etc.) of colonies developed on Petri dishes. Colonies were observed directly on a Petri dish under a microscope, at low magnification.

Morphological description of colonies was started from the fifth day of incubation and continued until the end of culture growth. At the same time preparations for microscopy were prepared.

The study of the finished preparation was based on the use of a dry optical system. For the identification of microscopic fungi guides were used (Bilayi and Koval, 1988; Malloch, 1981).

2.5 Biopreparation of endophytic microscopic fungi isolated from drought resistant plants:

Cultivation of selected endophytic microscopic fungi, isolated from xerophytes, was performed stationary, in thermostat at 30°C during 10 days, in 250ml volume conical vessels, in 50ml nutrient medium of the following composition (g/l): $NaNO_3$ - 9.0; KH_2PO_4 – 1.0; $MgSO_4 \times 7H_2O$ – 0.5; KCl – 0.5; $FeSO_4 \times H_2O$ – 0.02; glucose – 29; malt sprouts - 1,0; pH of the nutrient medium 5,5- 5,8; sterilization regimen - 121 °C, 15min.

After the cultivation was over, the content of each vessel was filtered through the glass filter and the supernatant was used as a liquid biopreparation.

2.6 Evaluation of the efficiency of biopreparation under the laboratory conditions

Pepper (*Capsicum annum* L.). - the annual agricultural plant was used to test the efficiency of the biopreparation under the laboratory conditions.

Free of chemical treatment seeds of the pepper were sowed in deep pots with sandy loam (one seed in one pot). On the 20th day of cultivation, at the three-leaf stage, plants were sprayed with biopreparation (2-3ml on each plant). Untreated plants served as control. There were 50 plants in each experimental variant.

To mimic the drought conditions watering of plants was ceased on the 40th day of cultivation, and water deficiency conditions were created; which continued for several days since the full drying of the soil.

The efficiency of the biopreparation was evaluated by the biometrical and biochemical indices of plants. For this purpose the habitus of both control and sptayed plants was observed and the height was measured; as well as the content of plastid pigments and ascorbic acid in leaves was studied.

2.7 Biochemical characteristics of experimental plants

Content of chlorophylls and carotenoids in leaves of studied plants was measured in 96% ethanol extract, spectrophotometrically. The optical density of the filtrate was measured on a corresponding vawelength on a spectrophotometer (SPEKOL 11, KARL ZEISS, Germany) and the concentration of pigments was calculated by the Vintermanns formula (Gavrilenko et al., 1975).

Ascorbic acid was determined in mg% by the titration method, with 0.0001N dichlorphenolindophenole solution (Ermakov et al., 1987). All analyses were performed with 3-fold repetition.

2.8 Statistical processing of data

Both, results of biometric and biochemical observations were processed statistically. One way ANOVA and Tukey's multiple comparison tests were used to test differences between means. All calculations were performed using statistical software Sigma Plot 12.5.

III. RESULTS AND DISCUSSION

3.1 Isolation of endophytic fungi from xerophytes

The composition of endophytic mycroflora significantly depends on plant species and is characteristic for the particular habitat where the given plant grows. The relations between plants and their endophytes are so symbiotic that the idea of their coevolution has been supposed (Hubbard et al., 2012). Accordingly, the possibility of the adaptation of xerophytes' mycobiota to extreme environment is very high. Based on this information it seemed advisable to isolate adapted to high temperature and water deficiency endophytes from xerophytes. For this reason a dry ecosystem at the suburb of Tbilisi, inhabited mainly with xerophytes was selected. 27 strains of microscopic fungi were isolated from above mentioned plant species and their pure cultures were received.

The first step in the identification of microscopic fungi was determination of the big-scale taxonomic unit, based on the structure and peculiarities of reproductive organs of a particular strain. Identification till the genus was performed according to morpho-cultural characteristics of a particular strain, following the guides (http://www.indexfungorum.org/, http://www.speciesfungorum.org/ https://www.mycobank.org).

TABLE 1
THE ENDOPHYTIC MYCOFLORA OF DROUGHT RESISTANT PLANTS

Plant species	Microscopic fungi
Artemisia lerchiana	*1. Penicillium* sp. GB 1-1 *2. Alternaria* sp.GB -1-2 *3. Aspergillus* niger GB 1—3
Erigeron canadensis	*4. Mucor* sp. GB 2-1 5. sp. GB 2-2 6. *Fusarium* sp. GB 2-3 7. *Alternaria* sp. GB 2-4 8. *Cladosporium* sp. GB 3-1 9. *Fusarium* sp. GB sp. 3-2 10. *Alternaria* sp. GB 3-3
Lactuca serriola	11.*Epicoccum* sp. GB 4-1 12.*Alternaria* sp. GB 4-2 *13.Fusarium* sp. GB 4-3 *14.Mucor* sp. GB 5-1 *15.Penicillium* sp. GB 5-2 16.sp. GB 5-3
Ballota nugra	17. sp. GB 6-3 *18. Alternaria* sp. GB 6-4
Polygonum aviculare	*19. Fusarium* sp. GB 7-1 20. sp. GB 7-2
Tribulus terrestris	*21. Alternaria* sp.GB 8-1 *22. Fusarium* sp.. GB 8-2
Zygophillum fabago	23. sp. GB 9-1 *24. Alternaria* sp.GB 9-2 25.sp. GB 9-3
Chenopodium album	26. sp. GB10-2-1 27. *Fusarium* sp. GB10-2

Isolated from grasses microscopic fungi belong to genera: *Alternaria*, *Aspergillus*, *Fusarium*, *Cladosporium*, *Mucor*, *Penicillium* and *Epicoccum* (Table1). The occurrence of these genera of microscopic fungi in the endophytic mycoflora of plants has been mentioned by other authors as well (Rashmi et al., 2019). Two strains of microscopic fungi were identified to the species, and 19 - to the genus as a result of the analysis. Seven cultures require additional molecular studies for identification (Table 1).

The mycoflora isolated from individual herbaceous plant was not distinguished by great diversity. Carroll (1988) pointed out the paucity of grass mycobiota even in the last century. According to his results one or two endophytes prevailed in the mycoflora isolated from a specific host, while other isolates were very rare. Later this fact was confirmed by other scientists (Arnold et al. 2003). Hyde and Soytong (2009) explained the small number of endophytic mycoflora by the fact that when endophytes are isolated by traditional methods on agarized food areas, fast-growing, "aggressive" cultures are isolated with a higher frequency, and the probability of "loss" of relatively slow-growing endophytes is very high (Hayd and Soitong, 2008).

3.2 Creation of consortia of endophytic fungi isolated from drought resistant herbaceous plants

To create a consortia of endophytic fungi of drought-resistant plants, adapted to high temperature and water deficiency was the main goal of the presented research. In order to select potential members of a consortium, selection of typical for the majority of tested plants cultures among endophytic mycoflora was decided; that is, the microscopic fungi reported as fast-growing "aggressive" cultures were to be revealed (Hayd and Soytong, 2008). Based on the calculation of the frequency of the individual genus of microscopic fungi in the endophytic mycoflora (Fig. 1), the dominant endophytes from the genera *Alternaria* and *Fusarium* were identified. It is significant to mention that these genera include many pathogens; but here following must be considered: generally fungi establish three types of relationships with the host plants: mutualistic (beneficial endophyte),

commensalistic (latent pathogen) and pathogenic (virulent pathogen). The type of relationship depends on the physiological status of the host, or the characteristic environment in which it is located. Depending on these three types of relationship, the fungus can enhance or reduce the life potential of the host, or remain neutral towards it (Alam et al., 2021). According to some scientists, there is no neutral relationship between the endophyte and its host, but rather a balanced antagonism. There is always a threat of aggression from the fungus, but the immunity of the host plant ties it up (Schulz and Boyle, 2005). This opinion is confirmed by the latest studies. It has long been known that *Alternaria alternata* is a ubiquitous species found in plants as an endophytic pathogen. Previously, it was believed that there were pathogenic and non-pathogenic endophytic forms of *Alternaria*. However, with modern molecular analysis methods, it has been determined that this is a single species that can be in a variety of nutritional relationships with the host plant (DeMers, 2022).

One microscopic fungus was also isolated from most of tested plants, which could not be identified at this stage of the study.

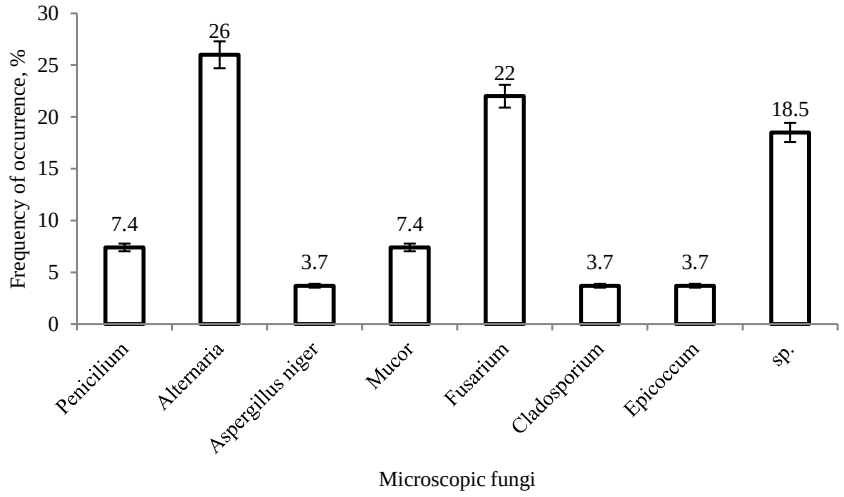

FIGURE 1: The frequency of occurrence of microscopic fungi spread among xerophytes

We assumed that mentioned dominant cultures were the fast-growing endophytes of the herbaceous plants of the selected location. To create a consortium, we focused on the dominant culture isolated from one specific species; in particular, into one consortium were grouped strains sp. GB 6-3 and *Alternaria* sp. GB 6-4, isolated from *Ballota nigra*; and in the second one we combined strains *Alternaria* sp. GB 8-1 and *Fusarium* sp. GB 8-2 isolated from *Tribulus terrestris*.

3.3 Testing the biopreparation of endophytic consortium on pepper plant in laboratory conditions

The suspension obtained by the submerged cultivation of a single strain of the consortium was used as a biopreparation. Three different biopreparations were prepared: 1) a mixture of suspensions of the cultural solutions of *Alternaria* sp. GB 6-4 and sp. GB 6-3; 2) a mixture of suspensions of the cultural solutions of *Alternaria* sp. GB 8-1 and *Fusarium* sp. GB 8-2; 3) the suspension of the culture solution of *Trichoderma viride* 12-1-1 - strain of the collection of microscopic fungi of the Faculty of Agricultural Sciences and Biosystems Engineering of the Technical University of Georgia.

Application of *Tr. viride* 12-1-1 (the strain which did not belong to the mycobiota of experimental xerophytes) was due to the fact that from the literature this microcopic fungus is known as a stimulator of plant growth and productivity and at the same time it stimulates plant drought resistance (Mona et al., 2017). Thus, it was interesting to test this endophytic fungus as well, in order to compare its effect with that of other experimental consortia.

3.4 Plastid pigments and ascorbic acid

The functional state of the photosynthetic apparatus is evaluated according to the content of photosynthetic pigments - chlorophylls and carotenoids (Lichtenthaller, Buschmann, 2001). It is known that water deficit, which is usually associated with intense illumination and high temperature, inhibits photosynthesis. One of the reasons for this is chloroplasts damage and chlorophyll destruction; which is even considered a kind of protective reaction to stress (Herbinger et al., 2002). Thus, the reduction of chlorophylls is a common phenomenon under drought conditions, which may be caused by the inhibition of pigment biosynthesis due to stress, or the degradation of pigments (Ma et al., 2020).

Carotenoids are the auxiliary pigments of photosynthesis with protective and structural function. Their photoprotective function is expressed by the effective neutralization of chlorophyll and oxygen excited molecules (Maoka, 2020). According to literary data by the carotenoids content one can discuss the stress intensity affecting plant, as well as the stress resistance of the latter (Strzalka et al., 2003).

Ascorbate is widely distributed low molecular antioxidant and important protective substance in plant. One of its principal roles is the protection of photosynthetic appatatus against oxidative stress (Venkatesh and Park. 2014). Increase of the content of ascorbate is one of the plant's primary responses to drought and intensive irradiation stresses (Yang et al., 2008).

According to all above mentioned we followed the changes in the content of carotenoids and chlorophylls in leaves of experimental plants under the drought stress, and studied the effect of the artificial inoculation with endophytic fungi on these indices. Investigation of ascorbate content as one of the key protective antioxidants of the photosynthetic apparatus was also interesting. The latter would give some information on the role of applied endophyts in stress-protection.

In pre-flowering phase the content of plastid pigments in experimental leaves was determined after three weeks of inoculation with endophytes. During this period experimental plants were subjected to artificial water deficiency for two times (pots were not watered until the turgor of leaves dropped significantly due to the lack of water, and the plants became "bored").

In flowering phase plastid pigments were determined in plants subjected to five–fold drought stress. In both phases of development, the content of chlorophylls and carotenoids in leaves was determined after the removal of stress (the plants were watered and the turgor of the leaves restored).

From the obtained results, it is clear that the content of chlorophylls in pre-flowering phase was statistically similar ($p>0.05$) in both experimental and control variants (Fig. 2). In the flowering phase, the difference between the test-variants appeared. In particular, the content of chlorophylls in leaves of the control variant was found to be lower than that of all test variants ($p<0.05$). Results were 1.6 times lower, compared to plants treated with the first consortium and 1.4 times less - compared to the variants treated with *Trichoderma* and the second consortium (Fig. 2). Between the test variants themselves a statistical difference was also revealed; in particular, the results of the first consortium-treated variant prevailed over the data with *Trichoderma*- and the second consortium- treated variants. It is interesting to note that the chlorophyll content in the first consortium-treated variant and *Trichoderm*a was statistically the same in pre-flowering and flowering phases, while in control and the second consortium-sprayed variant- decreased during the flowering phase (1.6 times and 1.2 times, respectively) (Fig. 2).

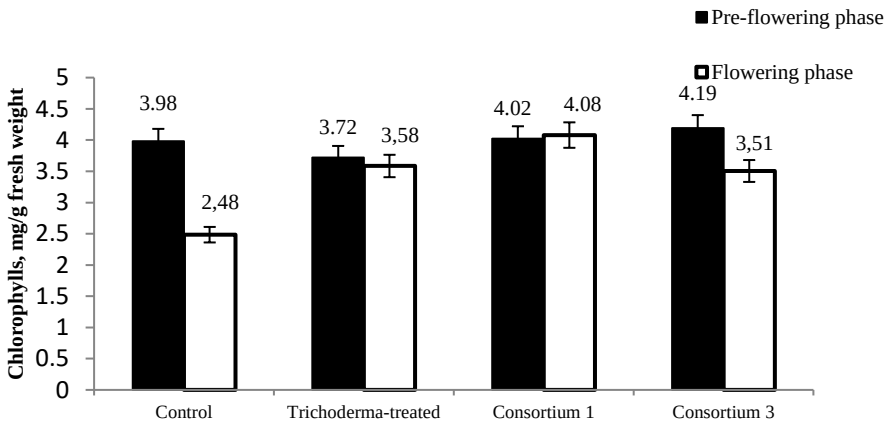

FIGURE 2: Content of chlorophylls in pepper leaves

As for carotenoids content, in pre-flowering phase a statistical similarity was observed between the control and the first consortium-treated variants ($p>0.05$), while the amount of pigments in the *Trichoderma*- and the second consortium-treated variants was higher compared to the control (1.9- and 1,7 times respectively) ($p<0.05$). Moreover, carotenoid synthesis was more active (by 10%) in *Trichoderma*-treated variant compared to the second consortium-treated one ($p<0.05$) (Fig. 3).

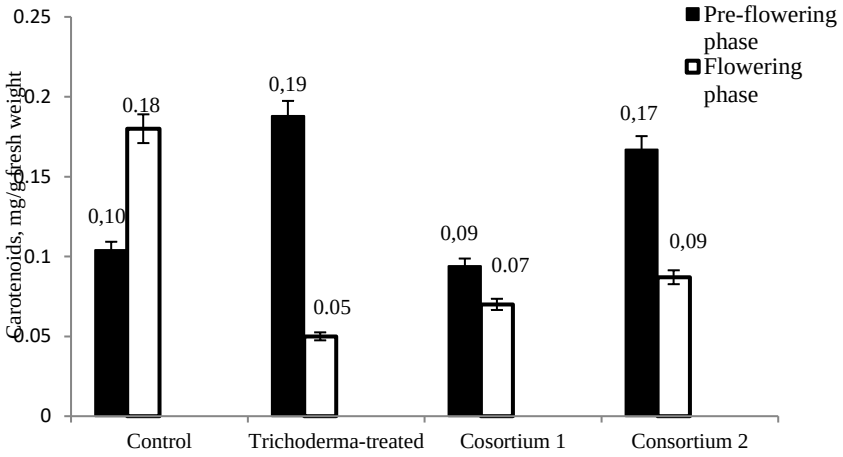

FIGURE 3: Content of carotenoids in pepper leaves

In flowering phase, the content of carotenoids in control and experimental variants was statistically different ($p>0.05$); moreover their content in control variant was significantly higher (1.8 times), compared to pre-flowering phase, and decreased in experimental variants: in the first consortium-treated variant - 1.3 times, in the second consortium-treated - 1.9 times; The content of carotenoids especially reduced in the *Trichoderma*-treated variant - 3.8 times (Fig. 3).

According to experimental results, it maybe assumed that inoculation with fungi increased the resistance of pigment system of experimental plants against drought stress. In particular, the first two "attacks" of water deficiency were not yet alarming for the plant, so the level of chlorophylls in the pre-flowering phase was the same in all tested variants. Along with the aggravation of the stress, probably the activity of endophytes also appeared. During the flowering phase, which is generally the most sensitive to stress, the chlorophyll content in the control variant decreased as a kind of adaptation to water deficit, while in the experimental variants its content remained almost at the level of the previous phase; that is, endophytes "relieved" the negative effect of water deficit on the pigment system so much that it did not change. Intensification of the stress pressure in the flowering phase caused a regular increase of carotenoids in the control variant; while the reduction of these pigments was noted in the inoculated variants. This can be considered as an indication that the pigment system of inoculated leaf was not particularly stressed.

What was the amount of one of the key compounds of the antioxidant system - ascorbic acid in leaves at that time? Its content in pre-flowering phase, under the influence of stress, was found to be lower in control plants compared to inoculated variants (Fig. 4). It should be assumed that the increase in ascorbate content is the “merit” of endophytes. Exacerbation of stress caused an increase of ascorbate in control variant (4-fold), which may be regarded as an adaptive response to stress. In treated variants, its content has changed little. In particular, in *Trichoderma*-treated variant ascorbate remained at the level of the pre-flowering phase (i.e. it did not change), while in consortia-treated variants it increased less compared to the pre-flowering phase (1.7-1.9 times); which may also be considered as an indication that the inoculated plants were not so acutely affected by stress as the control ones (Fig. 4).

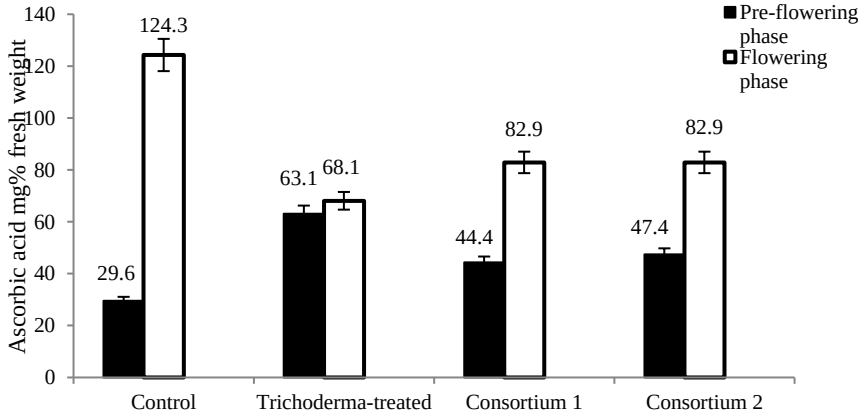

FIGURE 4: Content of ascorbic acid in pepper leaves

3.5 Sprouts development

The observation of pepper growth and development was begun after the spraying with biopreparations. Height of experimental plants was measured 4-times before flowering. The first measurement was carried out on the first day of spraying, when the effect of inoculation was not yet pronounced. These data were a kind of starting point for observing the changes in growth and development of experimental plants. Further measurements were taken at intervals of one week or more. In addition, the first two data were taken before exposure to artificial drought stress, the third - after the first stress, and the fourth - after three exposures to artificial drought.

According to the first measurement there were no statistical differences between the heights of different experimental variants ($p>0.05$); that is, sprouts growing under the same conditions developed equally. The second measurement revealed that the first consortium-treated plants were taller than all other options ($p<0.05$), and there was no statistical difference between them ($p>0.05$) (Fig. 5). The third measurement showed that the heights of the first and second consortium-treated plants were statistically close to each other ($p=0.3$) and prevailed over the control and *Trichoderma*-treated variants ($p<0.05$). By the fourth measurement, the first consortium-sprayed variant was still the leader in height ($p<0.05$) (Fig. 5).

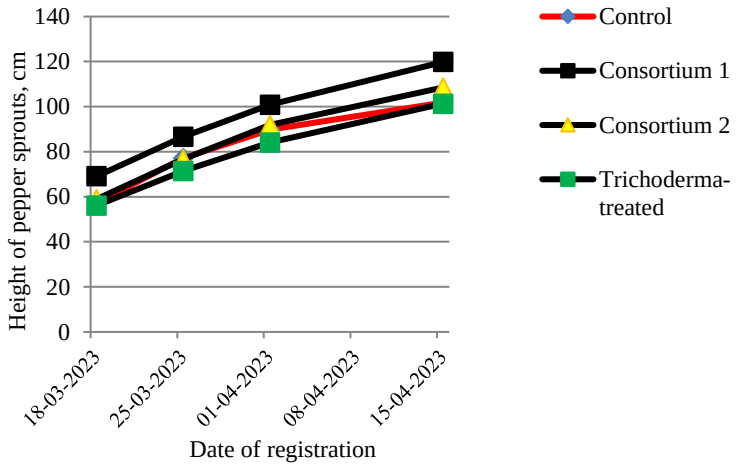

FIGURE 5: Dynamic of growth of pepper sprouts in pre-flowering phase

Based on the obtained results, it may be concluded that spraying with the first consortium had a stimulating effect on the growth and development of tested plants. The effect of the second consortium was relatively less pronounced.

Along with the growth stimulation, exposure to the first and second consortium also accelerated the transition of plants to flowering phase.

IV. CONCLUSIONS

1. Spraying pepper sprouts with biopreparations made from strains of microscopic fungi isolated from drought-resistant plants enhanced the drought stress resistance of experimental plants' photosynthetic apparatus.
2. Biopreparations stimulated the growth and development of test-plants and accelerated the onset of flowering.

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