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EFFECT OF EFFECTIVE MICRO-ORGANISMS ON THE PROLINE AND MDA CONTENTS IN HERB PLANT MATERIAL OF *OCIMUM BASILICUM* L. VAR. PICCOLINO

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ABSTRACT

Research on effective micro-organisms (EM) mainly focuses on their effect on crop yield and crop quality. On the other hand, knowledge on the effect of EM on metabolic processes that take place in plants at the cellular level is not sufficiently systematised. The subject of this study was to evaluate the effect of an EM preparation on two oxidative stress parameters, i.e. free proline and malondialdehyde (MDA) contents in the green parts of sweet basil *Ocimum basilicum* L. var. Piccolino grown in pots. The concentration of free proline was determined by the ninhydrin reaction and the malondialdehyde concentration based on the reaction with thiobarbituric acid. Analysis of variance for selected factors showed a significant effect of interaction both EM and time on the decrease of proline and MDA concentration. Effect of singular factor i.e. EM has shown a favourable influence of the preparation on the oxidative stress parameters in sweet basil by lowering the concentration of proline and significant slowing down the process of lipid peroxidation in the plant tissues. EM can be used in crop growing as a preparation to facilitate the adaptation of plants to changing climatic and habitat conditions.

KEYWORDS:
biochemical parameters, cultivation, oxidative stress reduction, sweet basil

INTRODUCTION

Due to the progressing environmental degradation as a result of crop chemization, the organic farming, natural fertilisers and preparations are becoming more popular. Effective microorganism (EM) technology consisting biological preparations composed of specially selected, naturally occurring microorganisms, is one of the alternative for modern agriculture[1, 2].

Various environmental factors cause changes in plants metabolism [3,4]. Prolonged or increased exposure to stress factor results in an imbalance between the generation of reactive oxygen species (ROS) and its antioxidant abilities, what in consequences can lead to plants death [5,6]. Proline and malondialdehyde (MDA) are good indicators of oxidative stress in plants [7-9].

Proline participates in the stabilisation of proteins and cell membranes [10,11]. It also serves as an osmoprotectant and is a reservoir of nutrients for plants [9]. MDA induces changes in the structure of the cell membrane leading to its disintegration and uncoupling of phosphorylation in the mitochondria [12]. Its concentration depends on the level of ROS in tissues – the greater the production of free radicals, the higher the concentration of malondialdehyde [13].

Scientific reports to date mainly focus on the evaluation of the effect of EM on crop yield and crop quality [e.g. 14-17] but do not explore the issues that concern the effectiveness of their protective properties based on the metabolic mechanisms taking place in plants at the cellular level. Most of this type research is conducted on crop plants. On the other hand, there are only few reports on herbaceous plants that enjoy the growing interest in Poland due to their high biological value. A very valuable herbaceous plant, of a wide range of application, is sweet basil (*Ocimum basilicum* L.) and its new varieties.

Therefore, the study determines the effect of EM on the level of free proline and MDA, the biochemical indicators adequate in the evaluation of biochemical activity and general physiological condition of sweet basil var. Picollino.

MATERIAL AND METHODS

Material. In 2014-2015, during the growing season, a two-year pot experiment with sweet basil (*Ocimum basilicum* L.) var. Piccolino was conducted. The plant material came from a private horticultural farm in Szczecin. Biochemical analyses on the acquired material were performed at the laboratory of the Department of Plant Physiology and Biochemistry, Faculty of Environmental Management and Agriculture, West Pomeranian University of Technology in Szczecin.

The experimental material was sweet basil var. Piccolino and EM. Sweet basil is an annual plant of the family *Lamiaceae*; the variety being tested is characterised by small leaves and a high content of essential oils. EM are natural preparations to support plant growth, being widely used in organic farming. They are composed of: lactic acid bacteria (*Lactobacillus casei*), photosynthetic bacteria (*Rhodopseudomonas palustrus*), yeasts (*Saccharomyces albus*), actinomycetes (*Streptomyces albus*) and mould (*filamentous*) fungi *(Aspergillus oryzae*).

A two-factor pot experiment was set up following the randomised complete block design in three replications. The first factor was 2 levels of EM application (level 1 involved the use of EM in cultivation, while level 2 is a control, without EM). The second factor was times of measurement (3 levels).

Sweet basil seeds, in the amount of 10 seeds per pot, were sown into a ready-made peat-based substrate with pH 5.5-6.5, salinity of 1.9 g $NaCl·dm^{-3}$ and with a starter dose of NPK compound fertiliser 14-16-18 in the amount of 0.6 $kg·m^{-3}$.

From the moment the seeds were sown, the objects intended for the application of effective micro-organisms were watered with an aqueous EM solution at a 1:100 dilution, every 7 days, in accordance to the manufacturer's recommendations. On other days, the plants were watered without addition of the EM preparation. On the other hand, the objects not intended for EM application were watered with plain water only.

The plant material for analyses was collected three times at monthly intervals, i.e. at the beginning of June, July and August. On all the dates, free proline and MDA contents in fresh herb parts of sweet basil were determined.

Proline (Pro) determination. The concentration of free proline in fresh green parts of sweet basil was determined by the ninhydrin reaction according to the method developed by Bates et al. [18].

Approximately 0.5 g of fresh plant tissue was homogenised in the presence of 3% aqueous solution of salicylic acid (10 ml), and the resultant homogenate was filtered through a filter paper. To the upper aqueous phase, 2 ml of acidic ninhydrin and 2 ml of glacial acetic acid were added. Next, the resultant solution was mixed thoroughly and, after pouring it into the closed tubes, placed in an incubator set at 90-100°C. After 1 hour, the tubes were transferred into an ice bath for 15 minutes to cool them. Then, 4 ml of toluene was added to each tube and they were shaken for 30 minutes. The samples prepared this way were left to allow the phases to separate. The upper phase (toluene) was sampled to determine the absorbance of chromatophore, against the blank, at the wavelength λ = 520 nm.

Malondialdehyde (MDA) determination. The concentration of malondialdehyde was determined by a slightly modified method according to Sudhakar et al. [19] that is based on the reaction of MDA with thiobarbituric acid.

The acquired plant material (1g) was homogenised with 0.1% TCA, then the resultant homogenate was filtered. To 1 cm^3 of the supernatant, 4 cm^3 of 0.5% TBA (in 20% TCA) was added. The closed tubes were placed in a water bath at 90-100°C and shaken for 30 minutes. Next, the tubes were placed in an ice bath for 15 minutes to cool them. The samples prepared this way were filtered once again, and then the absorbance against the reagent blank was determined in them at the wavelengths λ = 532 nm and λ = 600 nm. After removing the nonspecific turbidity being measured at λ = 600 nm, the MDA concentration was calculated using the mili-molar absorbance coefficient 155 $mM^{-1}·cm^{-1}$.

Both determinations were made using a Shimadzu 1800 UV-Vis spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, Md., USA).

Statistics. The findings with regard to the effect of effective microorganisms on proline and malondialdehyde concentrations in the plant material were subjected to a two-way analysis of variance (ANOVA). Homogeneous groups were determined by the Tukey's test at the significance level α = 0.05.

RESULTS

A significant effect of EM application on the concentration of free proline in the analysed plant material was shown (Tab. 1). The analysis of variance showed that the most significant statistical factor affecting the proline level in sweet basil herb was the time of taking measurements – 92.8 % (Tab. 1). The interaction of both factors, i.e. EM and time, had a significant effect on the analysed parameter – 4.7%.

Figures 1 and 2 present the effect of individual factors on the proline content. Under control conditions (without EM), the proline content was slightly higher than after EM application, the difference being however not significant. On the other hand, significant differences were found in the proline content depending on the time of measurement. The lowest proline concentration, ranging from 0 to 0.02 $\mu mol·g^{-1}$ f.w., was determined on the first and the second date of taking measurements for the two experimental variants. Its highest concentration was observed on the third date of taking measurements, regardless of the EM level, i.e. 1.48 $\mu mol·g^{-1}$ f.w. (Fig. 1).

TABLE 1
Analysis of variance for selected factors and interaction between the factors affecting the proline content.

Factor / interaction	SS	Df	MS	F	p	X
EM	0.11	1	107	11.45	0.005	1.22
Time	8.15	2	4074	436.13	0.000	92.80
EM x Time	0.41	2	207	22.11	0.000	4.70
Error	0.11	12	9			1.28

SS – sum of squared deviations from the mean, Df – degrees of freedom, MS – mean square (MS=SS/Df), F – F-test value, p – probability of error, X – percent effect of factors on the analysed property.

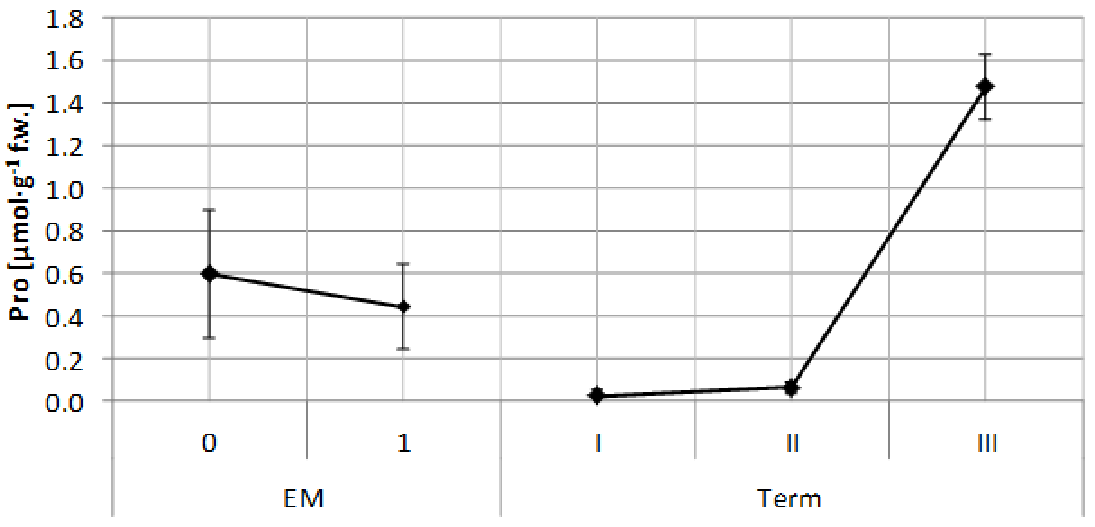

FIGURE 1
Average Pro concentrations in sweet basil herb for individual experimental factors.

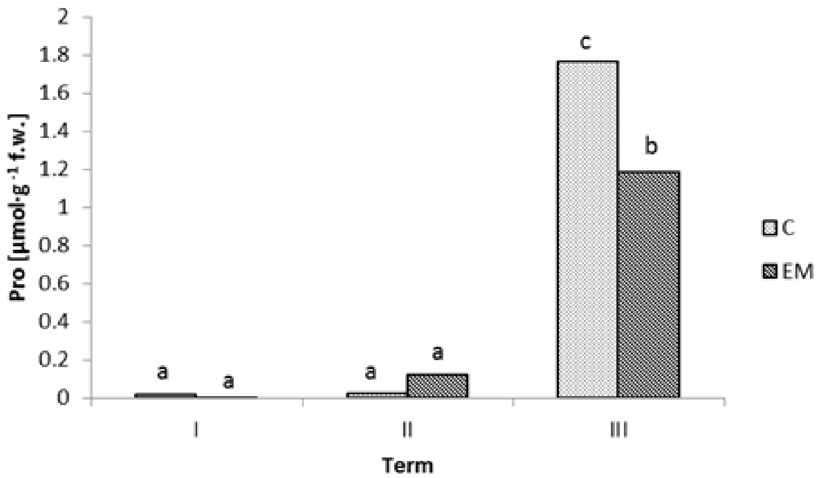

FIGURE 2
Pro content [µmol×g^{-1} f.w.] in the green parts of EM-treated sweet basil and under control conditions (without EM) depending on the time of measurement.

TABLE 2
Analysis of variance for selected factors and interaction between the factors affecting the MDA content.

Factor / interaction	SS	Df	MS	F	p	X
EM	130.07	1	130.073	361.45	0.000	22.89
Time	240.38	2	120.189	333.98	0.000	42.30
EM x Time	193.47	2	96.735	268.81	0.000	34.05
Error	4.32	12	0.360			0.76

SS – sum of squared deviations from the mean, Df – degrees of freedom, MS – mean square (MS=SS/Df), F – F-test value, p – probability of error, X – percent effect of factors.

Also on the third date of taking measurements – at the end of the growing season, a significant difference was found between the control plants (1.74 μmol·g^{-1} f.w.) and those with EM added (1.18 μmol·g^{-1} f.w.) (Fig. 2.). The EM addition decreased the proline content.

The EM preparation applied in the experiment also significantly decreased the malondialdehyde concentrations in the analysed plant material (Tab. 2.). The percent effect of this factor amounted to around 23%, with the time of taking measurements having once again the most significant effect on the MDA content in the plant tissue – 42.3%. The interaction of the two factors also showed a significant effect on the analysed parameter – around 34%.

Figures 3 and 4 present the effect of individual factors on the MDA content in the analysed material. The application of EM resulted in a 2-fold decrease in the MDA content in sweet basil herb compared to the control (Fig. 3). The time of taking measurements also significantly affected the average proline content in the analysed material. On the 1st date, its content was the lowest, whereas on the 3rd one the highest.

A significant interaction between the analysed experimental factors was observed (Fig. 4). The lowest MDA concentration was found in the plants collected on the 1st date and after EM application (3.17 nmol·g^{-1} f.w.), whereas by far the highest concentration was observed in the control plants collected on the 3rd date (20.22 nmol·g^{-1} f.w.).

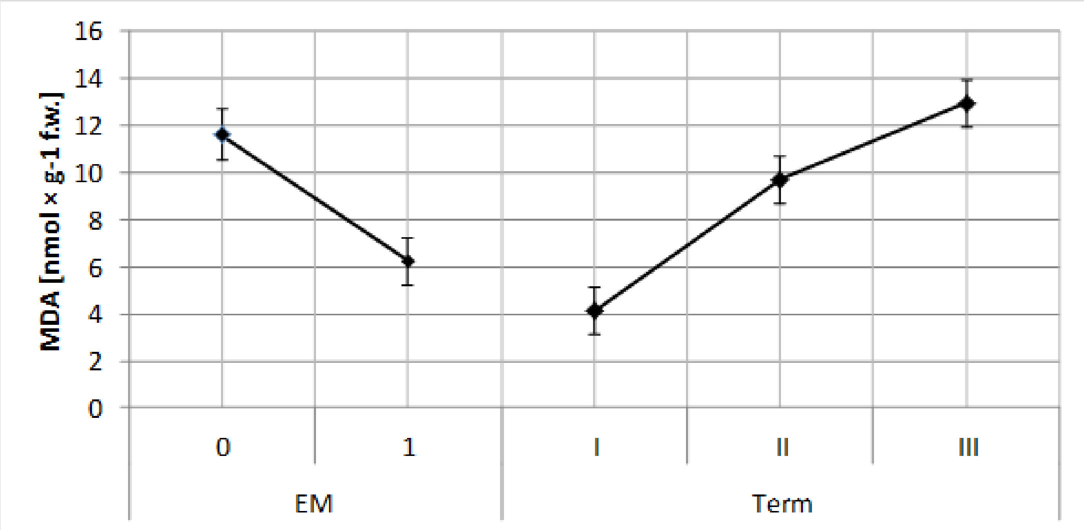

FIGURE 3
Average MDA concentration in sweet basil herb for individual experimental factors.

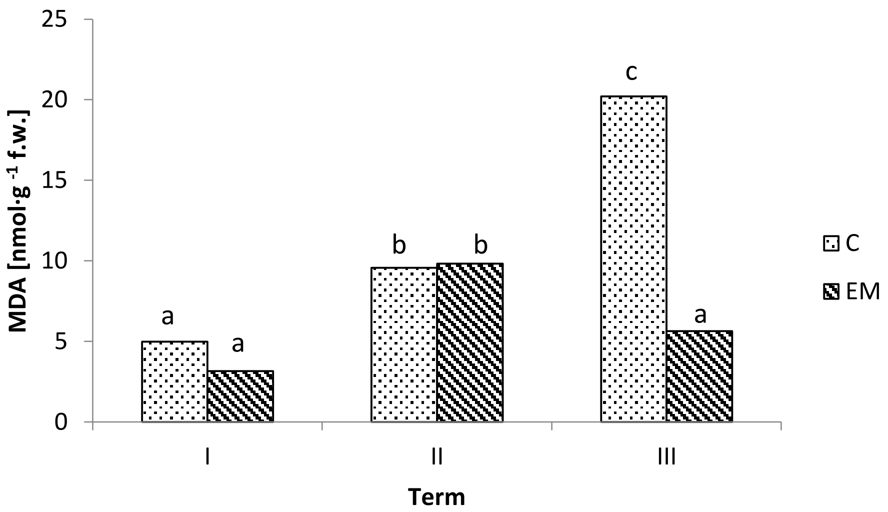

FIGURE 4
MDA content [nmol·g^{-1} f.w.] in the green parts of EM-treated sweet basil and under control conditions (without EM) depending in the time.

When comparing the MDA content in control plant herb and after EM application on respective dates of taking measurements, it was observed that the lowest concentration of this chemical compound was characteristic of the sweet basil plants being collected on the 1st date. However, the plants without EM contained significantly more MDA (5.01 nmol·g^{-1} f.w.), while those with EM less (3.17 nmol·g^{-1} f.w.). On the 2nd date, the MDA concentration increased and, regardless of the variant with EM, was at a similar level, i.e. 9.57 - 9.84 nmol·g^{-1} f.w. On the other hand, the MDA concentration in the control plants on the 3rd date was the highest (20.22 nmol·g^{-1} f.w.) and was significantly higher, 4-fold, than in the plants being treated with EM preparation (5.65 nmol·g^{-1} f.w.) – Fig. 4.

DISCUSSION

Due to the ongoing climate and habitat changes, the plants are exposed to the effects of abiotic factors that induce stress in them. To adapt to adverse conditions, the plants have developed some defence mechanisms that allow them to survive. Oxidative stress which is a response of the plant organism to the effects of stress-inducing stimulus is the phenomenon being most studied by scientists and best illustrates the condition of the test object. Bearing in mind the results presented in this paper, the significant increase of free proline content in sweet basil herb for the two variants of the material collected being collected on the third date should be taken into account in respect to other dates of making measurements. According to Koralewski [3], the proline level depends on both the internal environmental factors, such as plant age and its development stage, and the external ones, i.e. temperature, insolation, humidity, etc. Syversten and Smith [20] have demonstrated that the Pro content in the young plants is at the highest level than in the older ones – unlike in the present experiment. This may indicate the effects of a stress factor that activated the defence mechanism against free radicals. Since the function of proline includes, among others, osmoregulation, stabilisation of cell membranes and protection of plan tissues against degradation, the relevance of EM application in the analysed crop becomes essential. The obtained results show a significant reduction in the proline level in the plants being treated with EM preparation in relation to the control variant for the third date. Lower levels of this enzyme in the EM-treated plants may be justified by the presence of photosynthetic bacteria in the preparation which in co-operation with other micro-organisms provide plants with essential nutrients: amino acids, nucleic acids, bioactive substances and sugars [2]. The constant access to nutrients during stress allows protein degradation processes to slow down. In addition, EM are rich in micro-organisms that produce antioxidants, as well as in enzymes and hormones that support active cell division [1]. Talaat [2] in his experiment, has proven a mitigating effect of EM on the salt stress induced in common bean by increasing the protein synthesis and changing the composition of polyamines.

The effect of biologically active substances, i.e. bio-stimulators, on the proline content, other than that being observed in the present study, has been shown by Borowski and Blamowski [21]. They have observed a significant increase in the proline concentration in the leaves of *Ocimum basilicium* L. in the plants being treated with a bio-preparation compared to the control plants.

The MDA content in the analysed control variant is characterised by an upward trend over three months, which is consistent with the mechanism of organism aging. While on the first and the third date the MDA content in the EM-treated plants is significantly lower than in the control, the level of the analysed indicator on the second date seems to be striking. A similar level of this parameter for the two variants may indicate achieving optimum growing conditions and an adequate phenological phase by the plant in which the application of EM preparation does not bring significant changes. A significant reduction in the MDA level in the EM-treated plants in relation to the control on the third date points to the effects of antioxidants which, as reported by Higa [1], are the major product of EM.

A reduction in the values of oxidative stress parameters under optimum conditions as a result of the application of effective micro-organisms may be a confirmation of the protective properties of this preparation. According to Janas and Grzesik [22,23] biological conditioning of the seeds of some species of medicinal plants and vegetable crops enhances the health of seeds and improves their sowing value. On the other hand, Xu [24] and Chaudhry [25] have demonstrated the positive effects of the application of effective micro-organisms in maize growing. The bio-preparation has stimulated the growth of plants and induced their resistance and the process of photosynthesis [26].

CONCLUSION

The results obtained in this experiment confirm numerous scientific reports about the positive effect of EM on the growth and development of plants, not only under stress conditions. The study has shown a favourable effect of EM on the oxidative stress parameters in sweet basil by lowering the concentration of free proline and significant slowing down the process of lipid peroxidation in the plant tissues. Effective micro-organisms can be used in crop growing as a preparation to facilitate the adaptation of plants to changing climatic and habitat conditions.

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