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SHORT COMMUNICATION

GROWTH AND PROTEASE SECRETION BY ROOTS OF WHEAT SEEDLINGS CULTIVATED ON DIFFERENT NITROGEN SOURCES

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The aim of this paper was to study the interaction between protein and inorganic nitrogen use by *Triticum aestivum* seedlings cultivated on different variants of the Murashige-Skoog medium (MS): i) standard MS, ii) without inorganic nitrogen with 0.1% casein, iii) with 0.1% casein and inorganic nitrogen (5%, 25% or 100% of inorganic nitrogen from standard MS medium). The highest proteolytic activity in the medium and growth of wheat seedlings were obtained on the medium with casein and inorganic nitrogen. We assume that proteases secreted from wheat roots digested casein producing a pool of easy-accessible organic nitrogen and simultaneously applied inorganic nitrogen provided even higher biomass. Our studies suggest that the highest wheat yield can be obtained by usage of inorganic and organic nitrogen in adequate proportion.

Key words: **Plant nitrogen nutrition, protease exudation, *Triticum aestivum***

According to the actual stage of knowledge, plant roots are able to uptake not only inorganic nitrogen (IN) but also organic nitrogen (ON), namely: amino acids (Kielland 1994), di-, tripeptides and even oligopeptides (Tsay et al. 2007). However, opinions about the exact role of amino acids in plant nitrogen nutrition are diverse. Numerous studies have shown that the uptake of soil amino acids by plants was significant in spite of the microbial competitors (for example Näsholm *et al*. 2000). Critical view for this problem was also presented in studies, in which microorganisms strongly outcompeted plants (Jones *et al*. 2005). In addition, soil nitrogen is mainly present as proteins (Kaye and Hart 1997). Amino acids are released from soil proteins by digestion *via* proteases exuded by microorganisms (Nannipieri *et al*. 2002). In previous studies we showed that roots of higher plants also secrete proteases (Godlewski and Adamczyk 2007; Adamczyk *et al*. 2008).

In wheat fertilization inorganic nitrogen fertilizers are in use, but their excess can result in environmental pollution (Huang *et al*. 2003). Organic nitrogen fertilizers are also applied; their effectiveness is probably connected with the ability of wheat roots to take up intact amino acids (Näsholm *et al*. 2001). We already showed that casein can compensate the lack of inorganic nitrogen in the culture medium; this conclusion was based on the fact that wheat seedlings grew better on the MS medium without inorganic nitrogen but with addition of casein in comparison with standard MS medium (Adamczyk *et al*. 2008).

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The aim of this paper was to study the influence of diverse nitrogen sources on wheat growth and on the protease secretion into the culture medium. Seedlings were cultivated on different variants of the MS medium, having only inorganic nitrogen or only casein, or casein and 3 concentrations of IN - (5%, 25%, 100% from standard MS medium). After 3 weeks of cultivation seedling fresh weight and the proteolytic activity in the culture medium were measured.

Wheat seedlings (*Triticum aestivum* cv. Tacher) were grown from embryos isolated from grains. Plant material was obtained from Laboratory of Tissue Culture, Department of Biotechnology and Cytogenetics, Plant Breeding and Acclimatization Institute, Radzików, Poland. All chemicals were purchased from Sigma. Seedlings were cultivated like in previous studies (Adamczyk *et al*. 2008). Briefly, the embryos were germinated for 8 days on solid K_4NB medium (Kumlehn *et al*. 2006) and later seedlings were placed separately into tubes with 15 ml of medium: i) MS medium (Murashige and Skoog, 1962), ii) MS medium without IN with 0.1% casein, iii) MS with 0.1% casein and 5% IN (from standard MS medium), iv) MS with 0.1% casein and 25% IN, iv) MS with 0.1% casein and 100% IN. The seedlings were cultivated for three weeks. Germination and seedling cultivation were conducted in a controlled environment room at 23±1ºC air temperature, 70 % relative humidity, and 16:8 h photoperiod with 380 µmol m^{-2} s^{-1} light intensity at plant height.

The wheat grains were sterilized with 10% NaOCl for 20 minutes and washed with deionised water. Grains were kept at +4 ºC overnight and then sterilized again in the same way. Sterility in the culture medium prior to analysis was verified each time with a microbiological test, "microcount combi" (Schulke-Mayr).

Proteins from the culture medium were precipitated with $(NH_4)_2SO_4$ (70% of saturation). The precipitate was dissolved in 2 ml 0.9% NaCl in 0.05 M phosphate buffer (pH 6.0) and dialysed (Serva dialysis tubing) at + 4 ºC for 24 hours against the same buffer. Proteolytic activity was determined like in previous studies (Godlewski and Adamczyk 2007). Briefly, in first method (Anson 1938) to 0.5 ml of partially purified culture medium, 0.5 ml 2% (w/v) casein dissolved in 0.9% NaCl in 0.05 M phosphate buffer (pH 6.0) was added. After 4 h of incubation at 28 ºC the reaction was arrested by adding 2 ml 20% (w/v) TCA (trichloroacetic acid). After centrifugation, 0.5 ml of supernatant was mixed with 2 ml 6 % (w/v) Na_2CO_3, and then with 0.5 ml of Folin-Ciocolteau reagent (1/4, v/v diluted with deionised water). After 30 minutes, absorbance at 750 nm was read. In the control, TCA was added prior to casein. One unit of protease activity was defined as the amount of the enzyme that released from casein the amount of proteolysis products corresponding to 1 µmol of tyrosine during 1 h of incubation at 28 ºC.

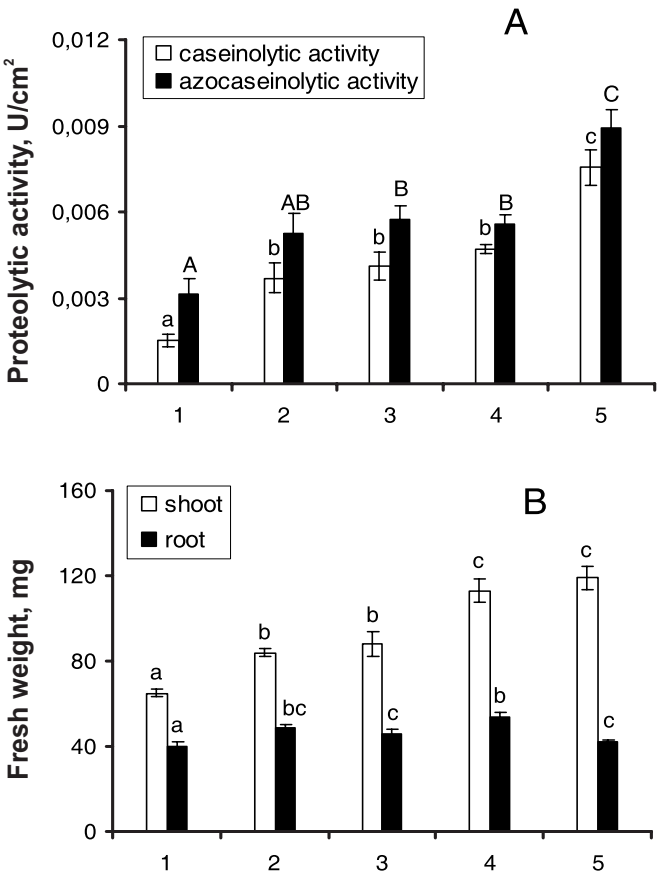

Fig. 1. A) Proteolytic activity in the culture medium and B) fresh weight and of seedlings cultivated on: 1 - MS medium, 2 – MS medium without IN with addition of 0.1 % casein, 3 - MS medium with 5% IN with addition of 0.1 % casein, 4 - MS medium with 25% IN with addition of 0.1 % casein, 5 - MS medium with 100% IN with addition of 0.1 % casein. Statistically significant differences (p<0.05) marked by different letters.

In second method (Tomarelli *et al*. 1949) to 2 ml of partially purified culture medium, 1 ml 0.5% (w/v) azocasein dissolved in 0.9% NaCl in 0.05 M phosphate buffer (pH 6.0) was added, and after 4 h incubation at 28 ºC, the reaction was arrested by adding 2 ml 20% (w/v) TCA. Then 1.5 ml of the supernatant was mixed with 0.5 ml 2 M NaOH, and after 30 minutes absorbance at 440 nm was read. In the control, TCA was added prior to azocasein. One unit of protease activity was defined as the amount of enzyme that increased the absorbance by 0.1 at 440 nm per 1 h at 28 ºC. Proteolytic activities are presented per mm^2 of the root surface, measured as described by Head (1966), on the basis of length of the root and root fresh weight.

Each experiment was performed in 6 replicates. The differences between variants (for both parameters – seedling fresh weight and proteolytic activity in the medium) were studied using ANOVA followed by Tukey's test. Correlations were calculated using the r-Spearman test. Statistical analyses were made using "Statistica" software (StatSoft Inc.).

Nitrogen fertilization is considered as important factor for increasing agricultural production (Shaaban 2006). Inappropriate usage of nitrogen fertilizers could not only decrease plant productivity and increase the cultivation costs but also could result in environmental pollution (Shi *et al*. 2007). Inorganic nitrogen fertilizers easily undergo leaching because of high mobility in the soil, especially nitrate (Jones *et al*. 2005) causing eutrophication of water reservoirs (Huang *et al*. 2003). Organic nitrogen fertilizers are more stable in the soil (Jones et al. 2005). Development of environmental-friendly methods of plant cultivation is an important factor in sustainable agriculture.

In this paper we studied the influence of diverse nitrogen sources on wheat growth and on the protease secretion from the roots. Like in our previous paper (Adamczyk *et al*. 2008), protein in the culture medium had positive effect on wheat growth, because root-secreted proteases digested proteins creating a pool of accessible organic nitrogen. In present studies, we showed that application of protein as the sole source of nitrogen resulted in lower fresh weight of seedlings than those growing on both sources of nitrogen (casein and inorganic nitrogen), and the same result was obtained in Paungfoo-Lonhienne *et al*. (2008) studies on sterile seedlings of *Arabidopsis thaliana*. We did not obtain significant difference between seedling growth on the MS medium without IN but with casein and on the MS medium with casein and 5% IN (from standard MS medium), which is in agreement with field studies conducted by Wang *et al*. (2004) in which application of low level of IN, in spite of abundant ON fertilization, did not result in high wheat growth. Moreover, lack of statistically significant difference between seedling growth on the MS medium with casein and 25% IN and those growing on MS with casein and 100% IN point to the fact that above certain concentration, higher amount of IN did not result in improving growth (similar results in field study of Shi *et al*. 2007). According to Wang *et al*. (2004) the proportion of IN to ON in wheat fertilization should be 2:1. In our studies, we used 0.1% casein (11.8 mM of N), and inorganic nitrogen in concentrations of 5%, 25%, 100% from standard MS medium (3 mM, 15 mM and 60 mM of N, respectively). We obtained the highest biomass for seedlings growing on MS with 0.1% casein with 25% of IN or with 100% IN, which means that proportion of IN to ON was about 1:1 and 4:1, respectively.

Positive correlation between IN concentration and the level of proteolytic activity (r=0.786, *P*=0.001 for azocaseinolytic activity) may suggest that IN could also modulate protease secretion as well. Increase of IN concentration caused the enhancement of protease secretion, that was probably caused by the feedback. Enhanced growth of seedlings cultivated on the medium with both sources of nitrogen (ON and IN) caused increase in protease secretion, leading to increase of a root-accessible pool of amino acids in medium.

Field studies are needed to confirm the importance of protease exudation for agricultural yield. In natural conditions also microbes exude proteases (Read 1991). However, organic fertilizers provide rich input of organic nitrogen. Such great amount of material can exceed digestive abilities of microbial proteases providing also substrates for plant root–secreted proteases. The use of inorganic and organic nitrogen may have great impact not only on agricultural yield but also on environmental protection.

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