RESEARCH ARTICLE

Inter-specific variability in protein use by two vegetable crop species

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Received: 04 May 2010; Accepted: 27 September 2010

ABSTRACT

It is now well-known that plants can uptake not only inorganic nitrogen but also organic nitrogen compounds, mainly amino acids. However, soil proteins are the main pool of amino acids. According to our earlier papers, plants can get access to this source of nitrogen using root-secreted proteases, but the level of proteolytic activity of such root-secreted proteases is species-specific. Our aim was to compare the use of protein as nitrogen source by two vegetable crops having high (*Allium porrum*) or low (*Lactuca sativa*) level of activity of root-secreted proteases. Seedlings were cultivated on Murashige and Skoog medium (MS), MS medium without inorganic nitrogen, MS medium without inorganic nitrogen, but with casein in concentration of 0.01%, 0.1% or 1%. Fresh weight of shoot of *A. porrum* was the highest for seedlings growing on culture medium with casein, but shoots of *L. sativa* obtained the highest weight growing on the culture medium with inorganic nitrogen. *Allium porrum* seedlings obtained 15-fold higher proteolytic activity in the culture medium than *L. sativa*. Seedlings of *A. porrum* using such high activity of proteases secreted by roots could provide a substantial pool of amino acids for intensive growth. The current studies conducted on *A. porrum* and *L. sativa* suggest that the efficiency of protein use in nitrogen nutrition by plants is species-specific.

Keywords: organic nitrogen, plant nitrogen nutrition, secretion of proteases

INTRODUCTION

It is now well established that plants can uptake not only inorganic nitrogen, but also organic forms of this nutrient, mainly amino acids, even in the presence of microbial competitors (Lipson and Näsholm, 2001; Ge et al., 2009; Näsholm et al., 2009), and especially in conditions of high amino acid concentrations in the soil (Jones et al., 2005; Sauheitl et al., 2009). The ability of plants to uptake amino acids was proven in numerous experiments for many plant species (i.e. Falkengren-Grerup et al., 2000; Persson and Näsholm, 2001; Xu et al., 2004; Hirner et al., 2006; Rentsch et al., 2007; Svennerstam et al., 2007). Amino acids are the major fraction of soil nitrogen; however, they are mainly present in polymeric form as proteins (Kaye and Hart, 1997). Protein utilization therefore is associated with proteolysis driven by microbial proteases. Our earlier studies showed, that also plants can secrete proteases by intact roots (Godlewski and Adamczyk, 2007) and that *Triticum aestivum* roots can directly utilize proteins in the culture medium as a source of nitrogen without prior digestion by microbial proteases (Adamczyk et al., 2008). This phenomenon was also proven by Paungfoo-Lonhienne et al., (2008); their studies were conducted on two non-mycorrhizal species - *Arabidopsis thaliana* and *Hakea actities*; additionally these authors showed that *Arabidopsis thaliana* growth was supported better by protein and low inorganic nitrogen than protein or low inorganic nitrogen alone. Moreover, Paungfoo-Lonhienne et al. (2008) suggested a possible uptake of intact protein by plant roots *via* endocytosis.

In our earlier paper we proved that proteases secreted by plant roots can digest proteins (casein, bovine serum albumin) to low-molecular-mass products (Adamczyk et al., 2009) which can be taken up directly by plant roots (Rentsch et al., 2007; Tsay et al., 2007; Paungfoo-Lonhienne et al., 2009). However, the level of proteolytic activity in the culture medium of different plant species varied significantly (Godlewski and Adamczyk, 2007). Also the uptake of amino acids – the product of proteolysis process - differs between various plant species (Weigelt et al., 2003, 2005; Okamoto and Okada, 2004; Reeve et al., 2009).

In this paper we were looking for a linkage between these differences in organic nitrogen uptake and the level of proteolytic activity in the culture medium. We were studying if there are some differences in the use of proteins by various plant species – *Allium porrum* cv. Bartek and *Lactuca sativa* cv. Ewelina. We have chosen these species because of high discrepancy between their root-secreted proteolytic activities in the culture medium in preliminary studies – *A. porrum* had the highest and *L. sativa* had the lowest proteolytic activity, comparing to other studied species (Godlewski and Adamczyk, 2007). These plant species were cultivated in sterile conditions on Murashige and Skoog medium (MS; Murashige and Skoog, 1962), MS medium without inorganic nitrogen, MS medium without inorganic nitrogen, but with casein (0.01%, 0.1% or 1%).

Our hypotheses were that (1) *A. porrum* seedlings would have higher shoot weight relative to *L. sativa*, growing on medium containing protein (2) *L. sativa* would be less effective at growing on protein compared to *A. porrum* seedlings (3) these variations in protein use could be reflected also in the level of proteolytic activities in the culture medium.

MATERIALS AND METHODS

Plant material and cultivation: Seeds of *Allium porrum* L. cv. Bartek and *Lactuca sativa* cv. Ewelina were obtained from a commercial plant distributor (Torseed, Poland). Plants were cultivated in aseptic conditions as in our previous studies (Godlewski and Adamczyk, 2007). Briefly, seeds were surface sterilized with 70% ethyl alcohol and with 10% sodium hypochlorite; and germinated on Petri dishes (7 days) and later separately in tubes (for 2 weeks; whole experiment took 3 weeks), containing 15 ml of autoclaved liquid medium: Murashige and Skoog medium (MS), MS medium without inorganic nitrogen, MS medium without inorganic nitrogen, but with casein in concentration of 0.01%, 0.1% or 1%. MS medium without modifications includes 60 mM of inorganic nitrogen, MS medium without inorganic nitrogen but with casein included: 1.18 mM, 11.8 mM and 118 mM of nitrogen for 0.01%, 0.1% and 1% casein concentration, respectively. The experiment was performed on liquid medium instead of sterilized soil, because soil contains both, inorganic and organic sources of nitrogen, so it would not be possible to obtain very controlled conditions of nitrogen sources. The seedlings were cultivated at 23 ± 1 °C air temperature, 70% relative humidity, and 16:8 h photoperiod with 380 μmol m-2 s-1 light intensity at plant height. Sterility in the culture medium after cultivation and purification was verified with microbiological tests (Microcount combi; Schülke-Mayr).

Proteolytic activity measurements: The culture medium was purified and concentrated using a Vivapore 5 solvent absorption concentrator (Sigma) with pore size 7500 MWCO. Proteolytic activity was determined using method by Tomarelli et al. (1949), in which the time-dependent release of azo dye-coupled trichloroacetic acid soluble peptide fragments from the substrate, azocasein, was monitored. Briefly, 200 μl of partially purified and concentrated culture medium was mixed with 100 μl 0.5% (w/v) azocasein dissolved in 0.9% NaCl in 50 mM phosphate buffer (pH 6.8). After 4 h incubation, the reaction was stopped by adding 200 μl 20% (w/v) trichloroacetic acid. After centrifugation (10000g, 5 min.), 150 μl of the resulting supernatant was mixed with 50 μl 1 M NaOH, and after 30 minutes absorbance at 440 nm was read (Hitachi U-2000 Spectrophotometer, Japan). In the control, trichloroacetic acid was added to the culture medium prior to addition of 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. All reagents were purchased from Sigma. Proteolytic activities are presented per one square millimeter of the root surface, which was measured with stereological method, as described by Head (1966), on the basis of length of the root and root fresh weight.

Azocasein method was chosen for this study because it is a nonspecific protease substrate, what means that azocasein can be easily digested by different proteases. Azocasein is often used for measuring the overall proteolytic activity (Hano et al., 2008). Methods of measurements of proteolytic activity on the basis of azocasein digestion are widely used (i.e. Zhang et al., 2007; Rojas et al., 2009).

Statistics: Each experiment consisted of 6 replicates (n=6). Proteolytic activity means were compared among nitrogen treatments and fresh weight means were compared among nitrogen treatments using one-way ANOVA, followed by Tukey's test. We used Statistica (Statsoft, Inc.).

RESULTS

Fresh weight of roots and shoots of *A. porrum* and *L. sativa*: Fresh shoot weight of *A. porrum* was the lowest (9.8 mg) on MS medium without any source of nitrogen and the highest shoot growth (32.8 mg) was obtained on MS medium in which inorganic nitrogen was replaced by casein (0.1% and 1% concentrations) ($P<0.01$) (Figure 1 A). Growth of *A. porrum* shoot was similar on standard MS medium compared to MS medium without inorganic nitrogen but with 0.01% casein ($P<0.99$). Fresh shoot weight of *L. sativa* was the lowest (11 mg) on MS medium without any source of nitrogen and the highest shoot growth (71.2 mg) was obtained on MS medium with inorganic nitrogen (Figure 1 B).

The lowest values of fresh weight of roots of *A. porrum* (2.7 mg) were obtained for seedlings growing on MS without nitrogen; the rest of medium variants gave higher results (from 5 to 6.5 mg), but similar to each other (no statistical differences) (Figure 1 A). The lowest fresh weight of roots of *L. sativa* (2.6 mg) was obtained for seedlings growing on MS without nitrogen, higher values were obtained for seedlings cultivated on standard MS medium and MS with 0.01% casein (6 and 8.2 mg, respectively), but the highest *L. sativa* root fresh weight was obtained for seedlings growing on MS with 0.1% or 1% casein (10.5 and 11.2 mg, respecitvely) (Figure 1 B).

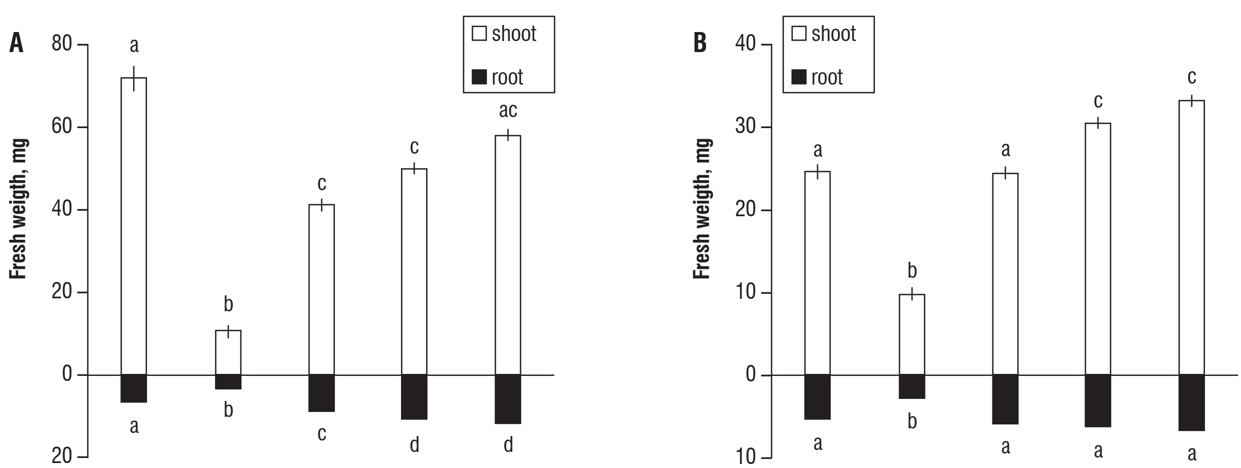

Figure 1. Fresh weight of *L. sativa* (1A) and *A. porrum* seedlings (1B) of seedlings cultivated on different media: (1) Murashige and Skoog medium, (2) MS medium without inorganic nitrogen, (3) MS medium without inorganic nitrogen, but with 0.01% casein, (4) MS medium without inorganic nitrogen, but with 0.1% casein, (5) MS medium without inorganic nitrogen, but with 1% casein. Values are means of six replicates. Error bars indicate standard error of the mean. Statistically significant differences ($P<0.05$) are indicated by different letters.

Comparison of the level of growth of *A. porrum* with that of *L. sativa*: To compare the level of growth of *A. porrum* and *L. sativa* we presented the results also in the form of percent of growth in comparison with growth on standard MS medium, which was treated as 100% of growth (Figure 2A). Inter-species comparison of shoot weight revealed that *A. porrum* seedlings obtained significantly higher level of growth on MS media with casein in comparison with the level of growth of shoot of *L. sativa* ($P<0.05$). In the same way we also presented root growth (Figure 2B). In comparison with root fresh weight of *A. porrum*, *L. sativa* roots obtained higher weight growing on media with casein (statistically significant for media with 0.1% and 1% casein).

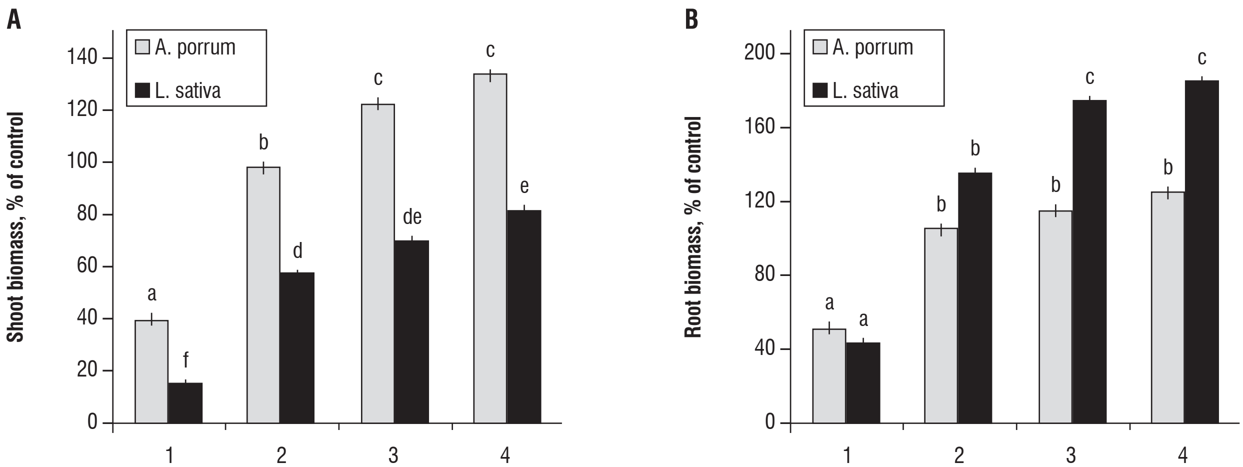

Figure 2. Comparison of growth of *Lactuca sativa* and *Allium porrum* shoot (2A) and root (2B) on different culture media: (1) MS medium without inorganic nitrogen, (2) MS medium without inorganic nitrogen, but with 0.01% casein, (3) MS medium without inorganic nitrogen, but with 0.1% casein, (4) MS medium without inorganic nitrogen, but with 1% casein. Results are presented as a percent of growth on standard MS medium (growth on standard MS medium was treated as 100%). Values are means of six replicates. Error bars indicate standard error of the mean. Statistically significant differences ($P<0.05$) are indicated by different letters.

Proteolytic activity in the culture medium: *Allium porrum* seedlings obtained 15-fold higher proteolytic activity than *L. sativa* (Figure 3). There were always statistically significant differences between activities obtained for *L. sativa* and *A. porrum* in each culture medium (not shown on figure). *Lactuca sativa* and *A. porrum* seedlings showed higher proteolytic activities growing on media with casein, especially in higher concentrations (for 1% casein concentration - 0.006 and 0.124 U cm^2 of proteolytic activity, respectively), in comparison with media with inorganic nitrogen (0.002 and 0.026 U cm^2 of proteolytic activity, respectively); but in the case of *A. porrum*, that increase in activity was greater.

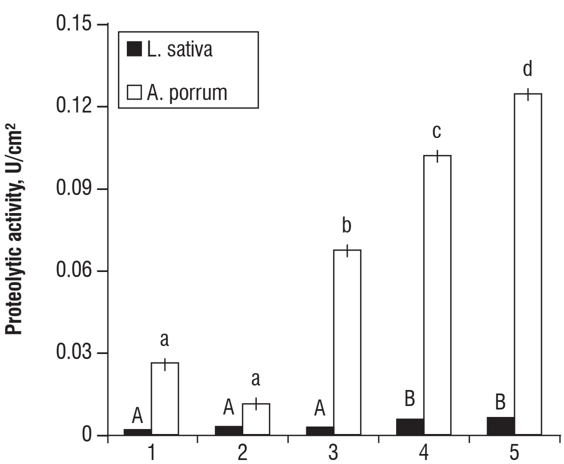

Figure 3. Proteolytic activity in the culture medium of seedlings cultivated on different media: (1) Murashige and Skoog medium, (2) MS medium without inorganic nitrogen, (3) MS medium without inorganic nitrogen, but with 0.01% casein, (4) MS medium without inorganic nitrogen, but with 0.1% casein, (5) MS medium without inorganic nitrogen, but with 1% casein. Values are means of six replicates. Error bars indicate standard error of the mean. Statistically significant differences ($P < 0.05$) between *A. porrum* media are indicated by different letters and significant differences and between *L. sativa* media are indicated by capitals. There were always statistically significant differences between activities obtained for *L. sativa* and *A. porrum* in each culture medium (not shown on figure).

DISCUSSION

There is increasing interest in developing ecological-friendly methods for plant cultivation. A primary means of attaining this goal is to decrease the use of inorganic nitrogen fertilizers, which contribute to environmental pollution (Huang et al., 2003; Wang et al., 2004). Plant assimilation of organic nitrogen (reviewed by Näsholm et al., 2009) provides a means for maintaining high plant yield and reducing environmental pollution. Plant species can differ in the ability to use soil organic nitrogen sources (i.e. Okamoto and Okada, 2004).

According to our earlier paper, plants growing in the same conditions can differ in the level of proteolytic activity in the culture medium, i.e. *Allium porrum* showed several fold higher proteolytic activity than *L. sativa* (Godlewski and Adamczyk, 2007). In this paper, we expanded that observation by the use of different variants on MS media (with inorganic or with organic nitrogen source). Both plant species (*A. porrum* and *L. sativa*) were able to use casein as nitrogen source and in the case of both species addition of protein to the culture medium resulted in significant increase of proteolytic activity. However, *A. porrum* was significantly more effective at growing on casein as a nitrogen source than *L. sativa*; such result was underlined by comparison of shoot growth of these two species as a percent of growth (growth on standard MS medium was treated as 100%) (Figure 2A). However, similar comparison of the level of root growth showed different pattern - roots of *L. sativa* obtained higher fresh weight than roots of *A. porrum*. Increased growth of roots in comparison with shoots can point to nitrogen deficiency (Robinson and Rorison, 1988; Ameziane et al., 1995) in *L. sativa* seedlings cultivated on medium with organic nitrogen.

Plants having higher proteolytic activity in the culture medium should show higher affinity to organic nitrogen sources in the form of amino acids - products of proteolysis. This is in accordance with paper by Matsumoto et al. (1999) in which authors showed that *L. sativa* prefers inorganic over organic nitrogen sources, and with Termine et al. (1987) studies, in which *A. porrum* plants were growing comparably on inorganic and organic nitrogen sources.

However, one should remember that these studies were conducted in laboratory conditions and in field conditions part of organic soil nitrogen does not exist in an easy accessible form, i.e. in complexes with tannins (Bending and Read, 1996). Moreover, tannins can not only create complexes with soil proteins – substrates for proteolysis, but tannins can also decrease proteolytic activity (He et al., 2006; Adamczyk et al., 2009).

According to our hypotheses, *A. porrum* seedlings obtained higher shoot fresh weight on medium with casein than *L. sativa*. Significant variations in proteolytic activities in different culture media (highest in *A. porrum* than in *L. sativa*, especially in the case of media with casein) point to mechanism hidden by these differences in plant growth - seedlings of *A. porrum* by using higher proteolytic activities in the culture medium than *L. sativa* were strongly increasing the pool of accessible organic nitrogen. Studies conducted on *A. porrum* and *L. sativa* suggest, that also other plant species can effectively use proteins in nitrogen nutrition; however efficiency of this strategy is species-specific.

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