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Research article

The ability of plants to secrete proteases by roots

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Abstract

The aim of our study was to find out if the culture medium of aseptically cultivated seedlings exhibits proteolytic activity and if this event is universal in angiospermous plants. Seedlings of 15 agricultural and wild-living plant species were cultivated for 14 days without any addition of nutrients. Our studies showed that roots of higher plants could secrete proteases and that levels of proteolytic activity in the culture medium of individual species (and cultivars of the same species) could be significantly different. The differences between quantities of the secreted proteases were connected neither with the fresh weight of the growing seedlings nor with the surface of the root system. No proteins were required to induce secretion of proteases. The culture medium of a few studied species (*Allium porrum*, *Zea mays*, *Helianthus annuus*) showed the highest proteolytic activity at pH 7. Studies of the influence of standard protease inhibitors showed that examined proteases belong to the cysteine protease family. The results suggest that the apical parts of roots exuded proteases more intensively than mature parts. Our studies suggest that some plant species could develop a strategy to actively increase the level of free amino acids in the soil solution as a source of N. Our results may contribute to studying plant N nutrition in natural ecosystems and to increasing yield after organic fertilization of agricultural species.

Keywords: Plant nitrogen nutrition; Protease activity; Roots; Secretion of proteases

1. Introduction

Nitrogen (N) is an important nutrient for plants and its concentration in available forms in soils is one of the limiting factors for plant growth [8,10]. Historically, it was assumed that plants can uptake only the inorganic form of nitrogen (NH_4^+ and NO_3^-) originating from mineralization of soil organic matter via soil microbial biomass [44]. Now, it is well known that plants of both natural and agricultural habitats can take up amino acids from soil, and these amino acids are potentially important N sources for plants (for review, see [24]). The uptake of nitrogen in the form of amino acids was shown in the case of plants growing in different natural ecosystems ranging from arctic and alpine tundra to the subtropical rainforest and ephemeral pools in the Namibian desert [1,22,29,35,38–40]. Amino acids as a source of nitrogen could also play an important role for agricultural plant species [19,30,44].

Species-specific preferences for using various sources of N point out that plants developed several strategies of using different forms of nitrogen, which are available in the soil solution. It was shown that the quantity of taken up amino acids depended on the concentration of free amino acids in the soil solution (e.g. [13,22]), on the type of amino acid [12,13,31,43], soil pH value [13], and competition between plants and soil microbes [5,17,18,20,25,26,33]. In plants, multiple sets of amino acid transport proteins that are specific or general in their transport of amino acids were identified [11,32]. These transporters can be used to take up amino acids from the soil and it is assumed that their expression pattern could be tightly regulated and species-specific [11,13].

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The release of free amino acids into the soil solution mainly results from the action of proteolytic enzymes exuded by microorganisms into the environment; these enzymes are also important factors promoting N cycle in the soil [4,28,42,45].

Plant root exudates include enzymes and other substances which increase the availability of nutrients taken up by plants from the soil [7,23]. Root exudates can also stimulate the development of rhizosphere microbes [8,14]. The question arises if plants can increase the free amino acid pool only indirectly by enlarging microbe populations in the rhizosphere, or if plant roots are also able to exude proteolytic enzymes. Such plant proteases would release amino acids independently from the activity of rhizosphere proteases secreted by microbes and could be especially important for those plant species which prefer amino acid N.

Exudation of significant quantities of proteases was suggested by our previous observations, which originated from the studies concerning a different problem. We observed that immersion of a fragment of a photographic film (exposed, fixed and developed) in the sterile medium of hydroponically cultivated seedlings could result in the digestion of the film emulsion after a few days of incubation. This effect was observed for several plant species studied. The ''photographic film'' method, which was used with over 30 agricultural plant species and cultivars, demonstrated a species-specific ability of plant root exudates to digest photographic film emulsion [2].

The aim of our present study was to test the ability of plant roots to exude proteases and to verify the prevalence of this event in angiospermous plants. We measured proteolytic activity in the culture medium of aseptically cultivated seedlings of several agricultural and wild-living plant species using biochemical methods. In the case of the chosen plant species we studied the correlation between the proteolytic activity and the time of seedlings growth. Moreover, we also measured the optimal pH value and the effect of protease inhibitors on these enzymes.

2. Materials and methods

2.1. Plant material

For this study 12 agricultural plant species (including two species in two cultivars) and 5 wild-living plant species were used. The former being *Allium cepa* L. cv. Wolska, (Liliaceae), *Allium porrum* L. cv. Bartek (Liliaceae), *Cucumis sativus* L. cv. Hela and cv. Julian (Cucurbitaceae), *Cucurbita pepo* L. cv. Bambino (Cucurbitaceae), *Helianthus annuus* L. cv. Paskowany (Asteraceae), *Lactuca sativa* L. cv. Ewelina (Asteraceae), *Phaseolus vulgaris* L. cv. Wiejska (Fabaceae), *Pisum sativum* L. cv. Iłówiecki (Fabaceae), *Raphanus sativus* L. cv. Murzynka and cv. Krakowianka (Brassicaceae) and *Zea mays* L. cv. Anawa (Poaceae) and the latter: *Geranium pusillum* L. (Geraniaceae), *Hippophae rhamnoides* L. (Eleagnaceae), *Ornithogalum umbellatum* L. (Liliaceae), *Ruta graveolens* L. (Rutaceae), and *Vincetoxium hirundinaria* L. (Asclepiadaceae). For our studies we selected cultivated plant species classified from various plant families, whose cultivation demands soil varying in fertility and nitrogen content and wild-living species from different terrestrial ecosystems.

2.2. Growth of plant material

The seeds were surface-sterilized by soaking in 70% ethanol for 2 min and then in 10% sodium hypochlorite with one drop of Tween 20 (per 100 ml) for 15 min and rinsed five times with sterile, deionised water. The seeds were germinated at 23 °C in autoclaved Petri dishes with two layers of moistened filter paper. The non-damaged seedlings of each plant species with about 10-mm-long roots were used in sterile, hydroponic cultures. The seedlings were separately placed into small tubes (30 mm × 80 mm) with 15 ml of autoclaved, deionised water (pH 6.8) on absorbent gauze (cotton) in such a way that only the bottom parts of the roots were immersed in water with the top 5 mm above the water surface. Then, each culture was placed into a bigger, covered tube (35 mm × 230 mm). All operations were done under aseptic conditions in a laminar airflow cabinet. The seedlings were cultivated in a controlled environment room 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 was verified each time with microbiological tests (microcount combi; Schulke-Mayr), which include medium suitable for growth of microbes. Aliquots which were free of contamination were used for analysis.

2.3. Purification of the culture medium

The culture medium was first centrifuged (3000 × *g*, 20 min), the supernatant was brought to 70% saturation with ammonium sulphate and then the aliquots were centrifuged (3000 × *g*, 30 min). Preliminary studies established that 70% saturation with ammonium sulphate precipitated all proteases. The precipitate was dissolved in 8 ml of 0.9% NaCl in 0.05 M phosphate buffer (pH 6.8) and dialysed (Serva dialysis tubing) at 4 °C for 24 h against the same buffer. Then the dialysate was brought to the initial volume (15 ml) with 0.9% NaCl in 0.05 M phosphate buffer (pH 6.8). These partially purified aliquots were used to measure the proteolytic activity.

2.4. Casein-hydrolyzing activity

The caseinolytic activity was determined by the modified Anson's method [3] in which the time-dependent release of tyrosine from the substrate, casein, was monitored. The reaction mixture consisted of 0.5 ml of the partially purified culture medium and 0.5 ml of 2% (w/v) casein dissolved in 0.9% NaCl in 0.05 M phosphate buffer (pH 6.8). After 4 h of incubation at 28 °C the reaction was arrested by adding 2 ml of 20% (w/v) TCA (trichloroacetic acid). The protein precipitation took place at 4 °C and then aliquots were centrifuged (3000 × *g*, 20 min). Then 0.5 ml of the supernatant was first mixed with 2 ml of 6% (w/v) Na_2CO_3, and then 0.5 ml of