

Effect of pH on α -Amylase Activity and Early Seedling Growth of Red Clover (*Trifolium pratense* L.)

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Abstract

The pH of the surrounding media is one of the environmental factors that can severely limited legume growth and development. We preliminary examined the effect of four pH levels of germination media (4, 5, 6 and 7) on α -amylase activity, seed germination and radicle length of red clover (*Trifolium pratense* L.) and traits correlations to evaluate the significance of obtained relationships in development of an screening method for pH tolerance in the earliest phases of plant growth. The pH of germination media significantly affected red clover α -amylase activity ($P < 0.01$), germination ($P < 0.05$) and radicle length ($P < 0.05$). The overall α -amylase activity was higher at 5 and 6 of pH than at pH 4 and 7. The activity of α -amylase at the same pH was most intensive during the first two days of germination. The highest seed germination percentage was found at pH 5. Length of radicle was higher at pH 5 and 6 than at 4 and 7. The occurrence of positive correlations ($P < 0.01$) among tested traits suggested that the α -amylase activity might serve as a parameter for the pH tolerance testing.

Keywords: legumes, α -amylase activity, germination, radicle length, pH

Introduction

The pH of the surrounding media is one of the environmental factors that can severely limited legume growth and development. As it influences on the availability of Mn, Al, Mo, and P (Taylor and Quesenberry, 1996), most of the studies on the effect of pH on legume seedling establishment were carried out by combining its effect with the influence of Al or Ca (Brauer, 1998; Brauer and Staley, 2005; Voigt and Staley, 2004). However, a few reports suggested that pH might affects legume growth and development independently of other environmental factors (Tanaka *et al.*, 1984; Yokota and Ojima, 1995).

The initial legume seedling growth begins after imbibition of water (Taylor and Quesenberry, 1996) when hydrolytic enzymes mobilize storage material accumulated in the starchy endosperm. Among these enzymes, the most abundant is α -amylase (Sultana *et al.*, 2000), which hydrolyzes the endosperm starch into metabolizable soluble sugars (MacGregor *et al.*, 1988; Sugimoto *et al.*, 1998). Changes in the activity of α -amylase of germinating plant seeds have been documented (Bienvenido and Varner, 1969; Dunn, 1974; Jones and Jacobsen, 1991; MacGregor and Matsuo, 1982; Sun and Henson, 1991). The results so far obtained have indicated that the α -amylase activity is under significant influence of variegated environmental conditions such

as temperature (Sultana *et al.*, 2000), aerobic/anaerobic conditions (Guglielminetti *et al.*, 1995; Guglielminetti *et al.*, 2000; Perata *et al.*, 1997), vanillic acid stress (Jazayeri *et al.*, 2007) or pH (Tripathi *et al.*, 2007). Although α -amylase has decisive role in seedling growth (Machaiiah and Vakil, 1981), information on the correspondence between α -amylase activity and seedling development under different pH of germination media is lacking. The data on the correspondence between initial seed germination processes and seedling development under variegated pH might enhance opportunity to develop fast and inexpensive screening method for pH tolerance.

The purpose of this preliminary examine was to determine the effects of pH on α -amylase activity, seed germination and radicle length of red clover and traits correlations in order to evaluate the significance of obtained relationships in development of an screening method for pH tolerance in the earliest phases of plant growth.

Materials and methods

Plant material and germination assay: The seed of red clover (*Trifolium pratense* L.) cultivar Rajah (DLF Trifolium, Denmark), provided by commercial distributor (Migra d.d., Zagreb, Croatia) in 2008, was used. Specified germination at seed certificate was 87%. Thousand seed

weight was 1.98 g. Seed samples were germinated in dark during nine days at the constant temperature of 7°C and four pH levels of germination media (4, 5, 6 and 7). Each combination of seed and pH treatment was represented by three replicates of 100 seeds. Growth chamber used was RU MED Type 3501 (Rubarth Apparate GmbH, Laatzen, Germany). Media for seedling growth was filter paper (500x500 mm, 90 g/m², 67N, Munktell and Filtrak GmbH, Bärenstein, Germany) moisturised by the 50 mL of tap water (ISTA, 2003). Water pH was adjusted by addition of 0.1 M HCl to desired pH. Seeds were transferred every 24 h to the new filter paper moisturised by the freshly prepared solutions. To prevent evaporation each sample was placed into sealed plastic bag. Number of germinated seed and radicle length were recorded on 9th germination day.

Extraction and assay of α -amylase: α -Amylase (EC 3.2.1.1) activity was tested in dry seed extract and in seed samples at intervals from 1 to 9th day of germination period (after 24-48-72-96-120-168-216 hours at germination). Seeds samples were harvested, frozen with liquid N₂ and stored at -80° C. Prior to analysis, hundred seeds from each treatment were grounded to a fine powder in a mortar using a pestle and then homogenized with 2 mL ice-cold buffer of 50 mM Tris-HCl (pH=7.5) containing 1 mM EDTA. The homogenate was centrifuged at 30 000xg for 45 min and the supernatant was heated with 3 mM CaCl₂ at 70 °C for 15 min to inactivate β -amylase, debranching enzyme, and α -glucosidase (Sun and Henson, 1991). The heat-treated supernatant was used as the enzyme source. The α -amylase was assayed by quantifying the reducing sugars (glucose equivalent) liberated from soluble starch using the method described by Bernfeld (1955). The heat-treated supernatant (0.3 mL) was added to 0.5 mL of 100 mM Na-acetate buffer (pH=6.0) containing 10 mM CaCl₂. Reaction was initiated by adding 0.5 mL 2.5 % soluble starch and incubated 15 min at 37 °C. The reaction was stopped by adding 0.5 mL of DNSA reagent (40 mM 3,5 - dinitrosalicylic acid, 0.4 M NaOH and 1 M K-Na tartarate). After the addition of DNSA reagent, the samples were heated in boiling water bath for 5 min and then allowed to cool down in running tap water. For the blank, the DNSA reagent was added prior to the substrate solution. After dilution with distilled water (up to 7 mL), the A₅₃₀ was measured (Model 6305 UV/Vis Spectrophotometer Jenway Ltd., Dunmow, England). The reducing sugar formed by enzymatic activity was calculated using a standard curve obtained with glucose (0-5 μ mol) (Guglielminetti *et al.*, 1995). One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μ mol of glucose from soluble starch per minute under the assay conditions. All measurements were performed in triplicates. Chemicals: 3,5 - dinitrosalicylic acid and other chemicals were of the highest purity, commercially available and provided by Sigma, St.Louis, MO (USA),

Merck, Darmstadt (Germany) and Kemika d.d., Zagreb (Croatia).

Data analysis: Germination percentage (GP) was calculated using equation: $GP = SG/SP \times 100$, where SG is number of seeds germinated and SP is number of seeds planted, for every replicated-pH treatment. Extension of radicle (mm) was determined on 25 randomly selected seedlings of each replication and pH treatment and the mean value was employed for data analysis. Cumulative activity of α -amylase was calculated as the activity-day summed over all previous sample intervals (Alvarez and Guerrero, 2000) for each replicated pH-treatment. Data analysis was performed by SAS Software procedures PROC ANOVA and PROC CORR (SAS Institute Inc., 2002-2003).

Results and discussion

Results of ANOVA indicated that the germination media pH significantly affected activity of α -amylase ($P < 0.01$), seed germination percentage ($P < 0.05$) and radicle length ($P < 0.05$). The revealed significance of pH effect support previously reported data of analyses carried out for strong pH impact on α -amylase activity profiles (Nielsen *et al.*, 2001) or earliest seedling growth of clovers (Evers, 1985).

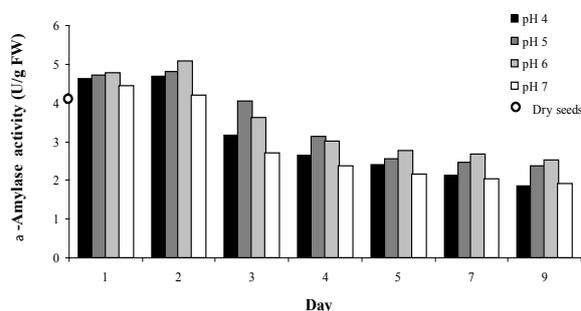


Fig. 1. α -Amylase activity of red clover under germination per days at different pH levels of germination media

The activity of α -amylase at the same pH value recorded daily indicated that activity was more intensive in dry seed extract and during the first two days of germination than after second day of germination, when activity rapidly decreased (Fig. 1.).

The obtained pattern of changes in α -amylase activity over days of germination was the same as patterns found by Kohno and Nanmori (1991, 1992) in alfalfa (*Medicago sativa* L.) and white clover (*Trifolium repens* L.). As red clover belongs to the same tribe as alfalfa and white clover, the result support Kohno and Nanmori (1992) suggestion that the pattern of α -amylase activity changes might be tribe specific. The cumulative activity of α -amylase was higher at 5 and 6 of pH than at pH 4 and 7 (Fig. 2. A, Fig. 2. B).

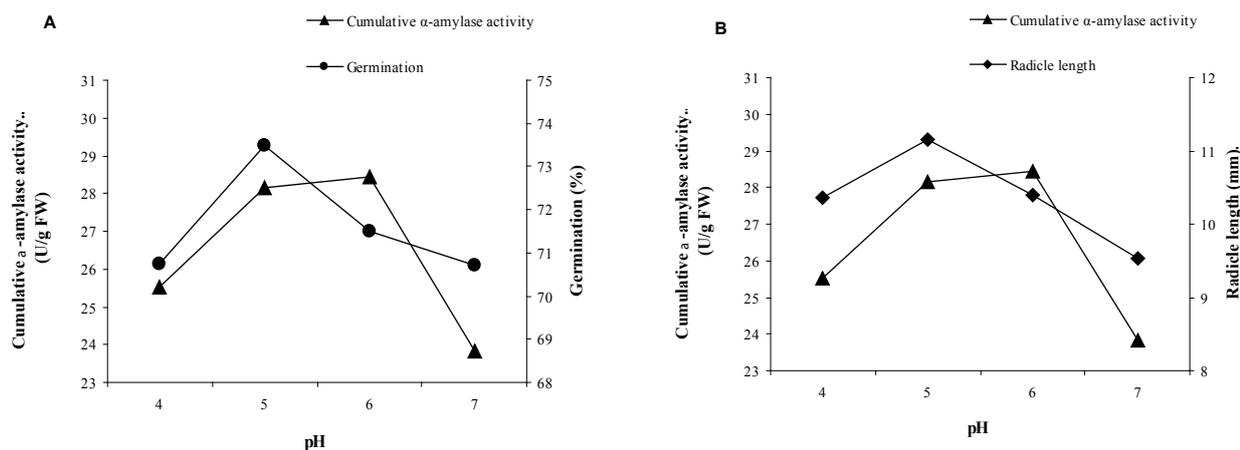


Fig. 2. Cumulative α -amylase activity and germination (A), radicle length (B) at different pH levels of germination media after 9 days of germination

The result corresponds to data on optimum pH for α -amylase activity evaluated on *Pisum sativum* L. (Beers and Duke, 1990), *Vigna radiata* L. (Tripathi *et al.*, 2007) and *Vigna angularis* L. (Mar *et al.*, 2003). The estimated cumulative α -amylase activity for the first 9 days of red clover germination ranged from 23.83 (pH 7) to 28.45 (pH 6) U/g FW. Seed was able to germinate at all tested levels of media pH. The result generally corresponded to a widely accepted opinion on fairly large environmental adaptation ability of red clover (Quesenberry *et al.*, 1991). As is shown in Fig. 2. A, the highest seed germination percentage was found at pH 5 (73.67%).

The obtained result indicated wider range of red clover pH tolerance than reported by Miller and Reetz (1995) (pH 6-6.5) and confirmed suggestion of Caddel *et al.* (2004) on optimal pH for red clover growth (pH 5-6). However, mean GP value across all pH treatments (71.67%) was lower than GP referred at official seed label (87%), probably due to a lower temperature (7° C) under which seed was germinated. Similarly, Hill and Luck (1991) reported reductions in rate of germination and increases in time of germination at lower temperatures for four *Trifolium* species examined. Moot *et al.* (2000) pointed out that the germination rates of the eight temperate herbage species tested increased linearly with temperature up to an optimum. Similar to cumulative activity of α -amylase, length of radicle was higher at pH 5 and 6 than on 4 and 7 (Fig. 2.B). The revealed variation indicated on importance of pH in seedling growth and establishment. Radicle length on 9th germination day varied from 9.1 mm at pH 7 to 11.2 mm at pH 5 in average. Cumulative α -amylase activity was positively correlated to both, germination percentage ($r=0.59^*$) and radicle length ($r=0.76^{**}$). The result was in agreement with previous analyses carried out by Jazayeri *et al.* (2007), who found positive correlation between activity of α -amylase and germination in rice, and Norastehnia *et al.* (2007) who reported negative correlation between root elongation and inhibition of α -amylase activity in maize. Obtained correlations suggested that the cumula-

tive α -amylase activity has potential to be early indicator for the legumes respond to pH stress. Germination percentage and radicle length were also positively correlated ($r=0.76^{**}$).

Conclusions

The results of this preliminary experiment has confirmed the importance of growth media pH to plant development and provided evidence for the occurrence of correlations between certain characters of red clover in its earliest phases of growth. The obtained significance of relationships suggested that the analysis of α -amylase activity might be useful initial step in development of fast and inexpensive screening method for pH tolerance. Further research on red clover cultivars and other small seeded legumes is currently underway.

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