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Diamine oxidase activity during the germinative and post-germinative growth of the embryonic axis in chickpea seeds

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Abstract

Diamine oxidase (DAO, EC 1.4.3.6.), which participates in oxidative catabolism of polyamines (PAs), was not detected in the dry viable chickpea (*Cicer arietinum* L.) seeds. From the time when the embryonic axis acquired an aerobic metabolism, DAO increased concomitantly with the growth of the embryonic axis and at the same time with the deterioration of the cotyledons, although in these organs the values were clearly lower than in the axis. The highest DAO activity in the embryonic axis of seedlings grown for 72 and 96 h was found in the elongation, differentiation and hypocotyl zones, while the lowest was in the apex and plumule. The absence of cotyledons promoted the early appearance of DAO in the embryonic axis. When germination occurred at supraoptimal temperatures (30 - 35 °C), DAO activity was sharply inhibited both in the cotyledons and in the embryonic axis. This inhibition was accentuated further in the presence of cyclohexylamine, an inhibitor of spermidine synthase activity, to such a degree that DAO was undetectable in the cotyledons. DAO inhibition by EGTA and the pronounced reversal induced by Ca^{2+} implies that calcium may be related to DAO activity. The presence of putrescine, spermidine and spermine in the germination medium stimulated DAO activity, although this activity was inhibited when the exogenous PA was cadaverine.

Additional key words: calcium, *Cicer arietinum*, cotyledon, cyclohexylamine, polyamine, thermoinhibition.

Introduction

Polyamines (PAs), a group of low molecular mass (M_r) polycations that are ubiquitous in nature, modulate many cellular processes (Galston and Kaur-Sawhney 1995, Minocha and Minocha 1995, Bhatnagar *et al.* 2001). PAs undergo major alterations during embryogenesis and germination (Matilla 1996, Gallardo and Matilla 1998) and also participate in flower and fruit development, morphogenesis, senescence or pollen development (Chibi *et al.* 1993, 1994, Galston and Kaur-Sawhney 1995, Matilla 1996, Kakkar *et al.* 2000). In plants, biosynthetic pathways of the principal PAs (putrescine, spermine and spermidine) have been described (*e.g.* Smith 1993), as well as the enzymes involved (*e.g.* Birecka and Birecki 1993). Putrescine (Put) may be synthesized from either ornithine or arginine, the former through the ornithine decarboxylase (ODC) pathway as found in animals, and the latter through the alternative arginine decarboxylase (ADC) pathway involving two intermediates, N-carbamoylputrescine and agmatine (Malmberg *et al.* 1998).

The oxidation of amines was recently reviewed (Medda *et al.* 1995), but little work is available on the oxidation of PAs during the growth and development of seeds. The cellular levels of PAs are regulated by anabolic and catabolic processes, oxidation being the only catabolic pathway known to degrade PAs (Smith 1985). In legumes (Smith and Barker 1988) this degradation pathway depends upon diamine oxidase

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Abbreviations: AdoMet - S-adenosyl-L-methionine; CHA - cyclohexylamine; Cad - cadaverine; DAO - diamine oxidase; PA - polyamine; PAO - polyamine oxidase; Put - putrescine; Spd - spermidine; Spm - spermine.
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(DAO) and, in *Poaceae*, polyamine oxidase (PAO). The activity of catabolic enzymes, especially in plants where DAO and PAO are very active, probably regulates the relative concentration of PAs. Immunological and histochemical studies have demonstrated that DAO is associated with the cell wall and membranes as well as with the apoplast, contributing to the polymerization of lignin and suberin (Smith 1990, Federico and Angelini 1991). The DAO located in the cell wall is inhibited by ozone (Peters *et al.* 1989). In maize plants, PAO is firmly bound to the cell wall, while in barley, it is symplastic (Li and McClure 1989, Federico and Angelini 1991).

In the present work, DAO activity during germination at optimal and supraoptimal temperatures and in different parts of seeds and seedlings was examined, comparing the results with the values previously found for PAs in this legume seed.

Materials and methods

Chemicals: Cadaverine (dihydrochloride), calcium chloride, cyclohexylamine, ethylene glycol-bis (β-amino-ethyl ether) N,N,N′,N′-tetraacetic acid, putrescine (dihydrochloride), spermidine (trihydrochloride) and spermine (tetrahydrochloride) came from *Sigma-Aldrich* (Madrid, Spain).

Seed germination: Seeds of chickpea (*Cicer arietinum* L. cv. Castellana), harvested in 1998, were purchased from commercial suppliers and stored in darkness at 4 °C until used. For germination experiments, seeds of uniform size were washed three times in sterile, double-distilled water, and then allowed to germinate in darkness at 25 °C (optimal conditions), or at 30 and 35 °C (supraoptimal conditions), and 70 % relative humidity for 96 h on top of two layers of sterile *Whatman No. 1* filter paper in plastic trays containing 50 seeds and 175 cm^3 distilled water (control). Depending on the experiment, the spermidine synthase inhibitor cyclohexylamine (CHA; 1, 10 and 100 mM), Spm, Spd, Put or cadaverine (Cad; 0.1, 1 and 10 mM) were added. Germination was scored as positive when the radicle tip had fully penetrated the seed coat (24 h). After removal of the seed coats, embryonic axes and cotyledons were aseptically separated, immediately frozen in liquid nitrogen and stored at -80 °C prior to DAO evaluation.

Growth of isolated embryonic axes: Seeds of uniform size were presoaked for 4 h in distilled water under continuous aeration, and the embryonic axes were then isolated intact according to Bueno and Matilla (1992). Samples of axes were grown under aseptic conditions in the dark (at 25 °C) from 6 to 96 h in 5 cm^3 of an autoclaved standard nutrient solution consisting of 10 mM Tris-HCl, pH 7.6, 20 mM KCl, RNase-free sucrose (10 g dm^{-3}) and 50 mg dm^{-3} chloramphenicol.

DAO extraction and determination: All extraction procedures were carried out on ice. Samples (1 g) of embryonic axes or cotyledons from *Cicer arietinum* seeds or isolated embryonic axes were homogenised in 100 mM potassium phosphate, pH 7.0, and centrifuged at 20 000 *g* for 30 min at 4 °C.

DAO (EC 1.4.3.6) activity was estimated in the supernatant, using an oxygraph (*YSI 5300, Hansatech,* Norfolk, UK) equipped with a Clark electrode (Rinaldi *et al.* 1982). The reaction, which contains the enzymatic supernatant (0.4 cm^{-3}) and 100 mM potassium phosphate, pH 7.0 (3.2 cm^{-3}), was carried out at 37 °C and was started by addition of 10 mM Put (0.034 cm^{-3}). As required, the specific calcium-chelant EGTA (5, 10, 25, 50 and 100 μM) or $CaCl_2$ were included in reaction cocktail. DAO activity was expressed as [nmol(O_2 consumed) mg^{-1}(protein) s^{-1}].

Distribution of DAO in different parts of the embryonic axis: To discover whether DAO activity was localized in different parts of the embryonic axis of chickpea seeds, the seeds were left to germinate for 72 and 96 h (25 °C), and the embryonic axis was cut into five sections: four from radicle (root apex, elongation and differentiation zones and hypocotyl) and the fifth corresponding to the plumule, as described in Gallardo *et al.* (1994). Each part was processed for DAO determination as described above.

Protein determination: Protein content was measured by the protein-dye-binding method of Bradford (1976) using bovine serum albumin as the standard.

Results and discussion

The germination of chickpea seeds (protrusion of the radicle) was delayed by temperatures lower or higher than 25 °C (Matilla 1996, 2000). This thermoinhibition has also been described in other seeds (*e.g.* Abeles *et al.* 1992, Bewley and Black 1994, Matilla 1996, 2000). During the germination of *Cicer arietinum* seeds, a major alteration occurs in the PA content, both in free form as well as bound to compounds of high and low M_r (Muñoz

De Rueda *et al.* 1993, Gallardo *et al.* 1996, Matilla 1996). The content of PAs are altered by germination (Muñoz De Rueda *et al.* 1993), temperature (Muñoz De Rueda *et al.* 1994), when channelling of S-adenosyl-L-methionine (AdoMet) towards the ethylene pathway was increased by inhibitors of PAs synthesis (Gallardo *et al.* 1994) and by the presence of exogenous PA_s (*e.g.* Put) in the germination medium (Gallardo *et al.* 1996). In this latter case, inhibition is notable in AdoMet-decarboxylase activity (Gallardo *et al.* 1996).

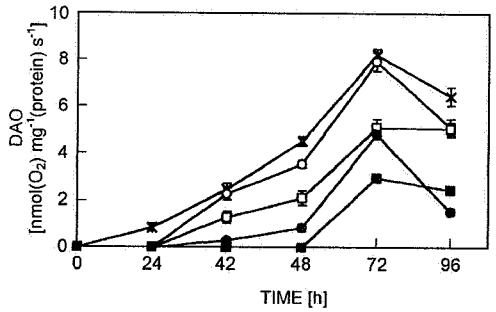

Fig. 1. Effect of supraoptimal temperature (30 °C) on DAO activity in embryonic axes (*open symbols*) and cotyledons (*closed symbols*) of chickpea seeds. Seeds were allowed to germinate at 25 °C (control; *circles*) and 30 °C (*squares*) and the enzymatic activity determined as described. *Crosses* indicates DAO activity in growing isolated embryonic axes (embryonic axis were isolated from dry seeds an allowed to grow at 25 °C during 96 h as indicated). Data are means of 3 - 4 different experiments ± SD.

The alterations in DAO activity during the germinative and post-germinative growth of *Cicer arietinum* seeds were studied under optimal (25 °C) and supraoptimal (30 - 35 °C) temperatures. The DAO activity was not detected in dry viable seeds nor in the embryonic axes or cotyledons of seeds during germination (24 h). Thus, it appears that DAO is necessary after radicle emergence, increasing progressively with post-germinative growth of the embryonic axis until reaching a maximum at 72 h. The profile of the alteration of DAO activity in cotyledons were similar to that in the embryonic axis but quantitatively lower (Fig. 1). Curiously, if the embryonic axes are grown in the absence of the cotyledon, the DAO activity was detected from 6 h of growth, and it is always greater than when it is determined from the embryonic axes within whole seeds (Fig. 1). The results in isolated embryonic axes can be due to absence of seed coat which is impermeable to O_2 (De la Fuente and Nicolás 1974). At 30 °C, the DAO activity sharply falls in the embryonic axis, and remains detectable in the cotyledon only after 48 h (Fig. 1). Similar to chickpea seeds, DAO activity is also absent from ungerminated soybean, pea and lentil seeds and appears early after germination (Suresh *et al.* 1976, Srivastava *et al.* 1977, Angelini *et al.* 1988). By contrast, in other plants, DAO activity is absent in the first phases of germination and then shows a rapidly increasing with the development of the seedlings (Cona *et al.* 1987, Torrigiani and Scoccianti 1995). Hirasawa (1988) showed that DAO develops in cotyledons as a result of the supply of O_2 through the embryonic axis, which controls this activity. From the data collected during different stages of germination of *Glycine max* and *Phaseolus vulgaris*, it has been hypothesized that DAO is involved in the regulation of the intracellular level of PAs (Scoccianti *et al.* 1990). The fact that during the first few hours of germination of chickpea seeds putrescine reaches a maximum in the embryonic axis at 12 h (Gallardo *et al.* 1992) may be explained by the absence of detectable DAO in that period (Fig. 1). However, other factors besides this enzymatic activity (*e.g.* transformation of bound into free PAs, preferential channelling of AdoMet towards PAs synthesis or transport of free PAs from cotyledons) may also be involved in the control of the level of this diamine precursor of Spd and Spm.

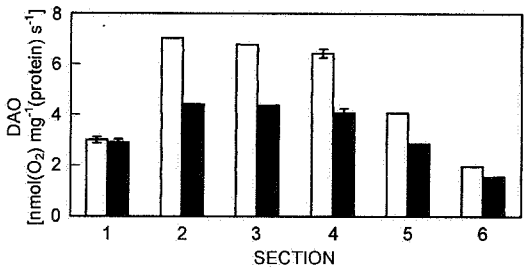

Fig. 2. Variation in the DAO activity over the length of the embryonic axis sectioned from seeds germinated in water (25 °C) for 72 h (*open bars*) and 96 h (*closed bars*). The results are the means of 3 - 4 diferent experiments ± SD. Radicle apex (1); elongation zone (2); differentiation zone (3); hypocotyl (4); plumule (5); residual cotyledon (6).

To study the distribution of DAO activity along the embryonic axis of *Cicer arietinum* seedlings, we germinated seeds for 72 and 96 h (25 °C) and divided their embryonic axis into 5 sections, as in Gallardo *et al.* (1994). In both periods of post-germinative growth, the highest specific DAO activity occurred in the elongation, differentiation and hypocotyl zones, and the lowest activity in the apex and plumule (comprised basically of meristematic cells). Both at 72 and 96 h, the residual cotyledon showed the lowest DAO activity (Fig. 2). The last-step of the ethylene biosynthesis pathway is very important in the hypocotyl, as is the accumulation of both free- and bound-Put and Cad (Gallardo *et al.* 1994). In *Phaseolus vulgaris*, DAO was found in young, actively growing parts (*e.g.* shoot apex), while in *Glycine max* it occurred mainly in elongating tissues (*e.g.* hypocotyl) in which exist high levels of Cad (Scoccianti *et al.* 1990).

A relationship between DAO and Ca^{2+} appears to be deduced from the present work (Fig. 3). The presence of EGTA, a selective Ca^{2+} chelator (*e.g.* Bethke *et al.* 1995),

in the DAO reaction cocktail, decreases enzymatic activity. This inhibition is correlated with the concentration of EGTA. This inhibition was almost completely reversed by exogenous $CaCl_2$. When Ca^{2+} was added to the reaction cocktail, the DAO activity was stimulated very slightly, perhaps because the content of endogenous Ca^{2+} in the supernatant from embryonic axes was sufficient for enzymatic requirements. An understanding of these results depends on the knowledge on the *in vivo* cellular location of DAO in the embryonic axis of chickpea seeds. Until now, DAO activity has been associated with the apoplast, cell wall and membranes (Li and McClure 1989, Peters *et al.* 1989, Smith 1993, Federico and Angelini 1991). DAO was not Ca^{2+} depend in pea seeds and the authors suggested that this divalent cation affected to the enzyme synthesis (Joseph and Srivastava 1995).

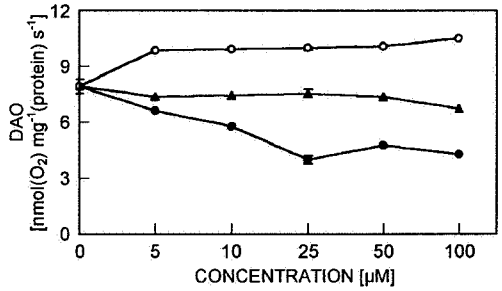

Fig. 3. Effect of $CaCl_2$ (*open circles*), EGTA (*closed circles*) and $CaCl_2$ + EGTA (*triangles*) on *in vitro* DAO activity. Seeds were germinated for 72 h (25 °C, water) and supernatant contained DAO activity obtained from embryonic axes as described in materials and methods. The results are the means of 3 - 4 different experiments ± SD.

PAs interact with other plant growth regulators, including ethylene. Thus, in some biological systems, when PAs synthesis is blocked by specific inhibitors, ethylene synthesis is promoted (Slocum and Flores 1991). This latter approach was taken into account to alleviate the thermoinhibition in *Cicer arietinum* seeds (Matilla 1996), in which germination depends on ethylene production by the embryonic axis (Matilla 2000). In embryonic axes of *Cicer arietinum* seeds, DAO activity was inhibited by supraoptimal temperatures (Fig. 4). With the addition of CHA, an inhibitor of spermidine synthase activity, DAO was inhibited with the increase in the concentration of the CHA both in control (25 °C) and at supraoptimal temperatures (30 and 35 °C) and at CHA (100 mM), DAO activity was not detected at any temperature. DAO activity was not detected in the cotyledons in the presence of CHA (data not shown). With the presence in the germination medium of Put, Spd and Spm at the concentration of 1 mM, the DAO activity was stimulated in both seedling axis and cotyledons (Table 1). However, the DAO was inhibited in the axis when the germination medium was enriched with Cad (1 mM). From these results we can conclude that the more effective polyamine with respect to DAO activity in *C. arietinum* seedlings was Put (65 % stimulation), followed by Spd (60 %) and Spm (40 %), while Cad proves an inhibition.

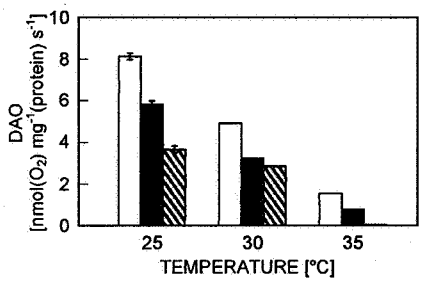

Fig. 4. Effect of CHA at zero (*open bars*), 1 mM (*closed bars*) and 10 mM (*striped bars*) on DAO activity from embryonic axes of chickpea seeds germinated for 72 h at different temperatures. The results are the means of 4 - 5 different experiments ± SD.

In previous works, we demonstrated that CHA, at optimal and supraoptimal temperatures inhibits the contents of Spd and Spm in the embryonic axis of *Cicer arietinum,* concomitantly increasing the contents of Put (Gallardo *et al.* 1994, Muñoz De Rueda *et al.* 1994). However, this important increase in Put content induced by the presence of CHA, do not provoke a stimulation of DAO. By contrast, the presence of exogenous Put (1 mM) in the germination medium stimulates DAO activity (Table 1), this stimulation being inhibited by addition of Put plus CHA (data not shown). Susuki (1996) demonstrated that CHA is a potent inhibitor of DAO activity in wheat seeds. Nevertheless, we cannot dismiss the possibility that Put induced by CHA is compartmentalized in places within the cell far from DAO and therefore would not have an capacity to alter DAO. This would not be the case of exogenous Put (Table 1) that penetrates from the germination medium because it easily enters to the apoplast, the probable location of DAO in legumes (Smith 1993).

Table 1. Effect of Put, Cad, Spd and Spm (1 mM) on DAO activity [nmol(O_2) mg^{-1}(protein) s^{-1}] from embryonic axes and cotyledons of chickpea seeds germinated at 25 °C for 72 h. Means of 3 - 4 different experiments ± SD.

	Control	Put	Cad	Spd	Spm
Embryonic axis	8.2 ± 0.2	13.3 ± 0.2	6.3 ± 0.4	12.0 ± 0.6	11.7 ± 0.8
Cotyledons	1.7 ± 0.1	5.0 ± 0.4	2.5 ± 0.3	3.3 ± 0.1	2.5 ± 0.2

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