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Original article

Variation for polyphenol oxidase activity in stems of *Medicago* species

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Abstract – Polyphenol oxidase (PPO) activity was detected in stems of both glandular-haired and glabrous wild *Medicago* species. Two PPO isoforms appeared in each *Medicago* species, the heavier isoform ranged from 120 to 180 kDa and the lighter one from 65 to 75 kDa. The substrate affinity for 4-methyl catechol was quite low (K_m around 0.5 mM) in all the species examined. The PPO specific activity present in the stem epidermis of insect-resistant *Medicago* spp. was remarkably higher than that measured in the same tissue of susceptible alfalfa, thus suggesting a possible role of PPO in resistance against biotic stresses. (© Inra/Elsevier, Paris.)

insect resistance / *Medicago* spp / Leguminosae / polyphenol oxidase

Résumé – L'activité de la polyphénoloxylase (PPO) dans des tiges d'espèces de *Medicago*. L'activité de la polyphénoloxylase a été détectée dans des tiges d'espèces spontanées de *Medicago*, aussi bien du type à poils glandulaires que du type glabre. Deux isoformes de PPO sont apparues dans chacune des espèces de *Medicago*, la plus lourde allant de 120 à 180 kDa et la plus légère de 65 à 75 kDa. L'affinité du substrat pour le 4-méthylcatéchol est restée sur des valeurs plutôt basses (K_m aux alentours de 0,5 mM) chez toutes les espèces examinées. L'activité spécifique de la PPO relevée dans l'épiderme de la tige des espèces résistantes aux insectes est apparue comme nettement plus forte que celle mesurée dans le même tissu chez la luzerne sensible, ce qui suggère que la PPO pourrait jouer un rôle dans la résistance aux stress biotiques. (© Inra/Elsevier, Paris.)

résistance aux insectes / *Medicago* spp. / Leguminosae / polyphénoloxylase

1. INTRODUCTION

Polyphenol oxidases (PPOs) are copper enzymes that, through the reduction of molecular O_2 , catalyse the hydroxylation of monophenols to *o*-diphenols (cresolase activity, EC 1.14.18.1) as

well as the oxidation from *o*-diphenols to *o*-quinones (catecholase activity, EC 1.10.3.2). PPOs are localised in the plastids and are widespread among the angiosperms but their biological functions have not been clearly established [22]. Generally, these enzymes are involved in the browning of fruits and tissues as a consequence of

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quinone adduct formation. For this reason they have been intensely studied in food technology [15]. Plant quinones also affect plant–pest interactions decreasing the nutritive value of plant proteins [6] and giving rise to a general toxic effect [16]. Another aspect of plant–insect resistance involving PPO activity concerns the O₂-requiring polymerisation of the exudate of glandular trichomes in some solanaceous species, which results in entrapment of insect pests [1, 12, 25]. Antibiotic factors against insect attacks have proved to be present in the exudate of glandular trichomes of several *Medicago* species, and their mechanism is supposed to consist of both toxic and mechanical anti-feeding features [23]. Glandular-haired *Medicago* spp. expressing insect resistance are generally wild and annual, while the insect-susceptible cultivated alfalfa (*Medicago sativa* L.) lacks glandular trichomes.

We measured the PPO activity in stem tissues of both glandular-haired and glabrous *Medicago* spp. in an attempt to correlate it with insect resistance. For this purpose the wild and completely autogamous *Medicago* spp. *M. rugosa*, *M. scutellata* and *M. intertexta*, showing various degrees of resistance to the most common alfalfa insect attackers, were taken into account [23]. Since the *Medicago* glandular trichomes did not brake upon contact, we compared the PPO activity of the stem epidermis of glandular-haired and glabrous races of *M. intertexta* to investigate trichome-specific PPO variants at both quantitative and qualitative levels. Susceptible *M. sativa* was also used as control.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds were obtained from the following sources: *M. rugosa* (SA 2202), *M. scutellata* (SA 18640), glabrous *M. intertexta* (SA 5788) were from the South Australian Department of Agriculture, Medicago Genetic Resource Centre, Adelaide, Australia and glandular-haired *M. intertexta* (242) was from the International Centre for Agriculture Researches in Dry Areas (ICARDA), Aleppo, Syria; alfalfa seeds belonged to the commercial variety 'Maremmana'. Five plants of each species were raised in the greenhouse.

2.2. Protein extraction and PPO assay

Epidermis was separated from stems with tweezers. Four hundred milligrams of either peeled stems or epidermis were ground in a mortar with liquid nitrogen, and the powder obtained resuspended in 500 µL of extraction buffer (50 mM sucrose; 100 mM NaPi, pH 7.0; 10 mM cysteine) [1]. The slurry was then centrifuged (15 000 g for 20 min at 4 °C) to pellet impurities and the supernatant was transferred to an eppendorf tube and kept on ice. Protein concentration was measured as described by Bradford [2]. PPO activity and enzymatic units were determined according to Esterbauer et al. [7] by using 10 mM 4-methyl catechol as substrate. Catalase (280 units, Sigma) was added to the assay solution to prevent oxidation of the substrate by peroxidase [22]. Change in A₄₁₂ was detected in a Varian Cary spectrophotometer.

2.3. PAGE gel staining

Proteins were analysed by PAGE under non-reducing conditions on 7.5 % polyacrylamide gels [18]. Gels were run at 25 V/cm for 4 h at 5 °C. After electrophoresis gel plates were stained for PPO activity in 0.1 M NaPi, pH 7.0, 0.3 M 4-methyl-catechol and 90 mM *p*-phenylenediamine for 5–10 min [1]. The PPO inhibitor diethyldithiocarbamate (DEDTC) was added to the staining mixture according to Sherman et al. [22]. M_rs were estimated by running gels of different acrylamide concentrations, from 4 to 12 %, and by calculating the protein mobilities as R_f. Proteins of known molecular weight (from 340 000 kDa, α₂-macroglobulin, to 26 600 kDa, triosephosphate isomerase) were electrophoresed under these conditions and the data obtained were treated as indicated by Hedrick and Smith [8] to create a standard curve.

3. RESULTS AND DISCUSSION

The method developed by Esterbauer et al. [7] which depends on following spectrophotometrically the decrease in the adsorbance of the yellow-coloured 2-nitro-5-thiobenzoic acid (TNB), was used to assay PPO activity. Enzyme activity was detected in all the *Medicago* species examined (table I) but not in boiled extracts. PPO affinity for 4-methyl-catechol was relatively low in epidermis of all the species investigated (K_m ca. 0.5 mM, table I). These values are in agreement with the K_ms reported in plants, usually around 1 mM [16], even if higher values have also been reported [20]. Table

I shows that PPO activity was higher in the epidermis than in peeled stems for all the species examined, but the ratio between the PPO specific activity of the epidermis and peeled stems was markedly higher in wild species (range 3.6–7.6) than in *M. sativa* (1.4). PPO specific activity present in the epidermis of insect-susceptible *M. sativa* was remarkably lower than that of all the annual resistant *Medicago* spp. However, PPO specific activity of the epidermis of glabrous *M. intertexta* was higher than that of its glandular-haired counterpart indicating that at least for this species, the high value of PPO specific activity of the epidermis is not necessarily correlated with the presence of glandular trichomes. Taken together, these results give a strong indication that PPO may be involved in resistance mechanisms in *Medicago*. On the other hand, increased level of PPO in tissues injured by insect attack has already been reported in alfalfa by Jiang and Miles [10]. Resistance to insect oviposition and larval development among annual *Medicago* spp. was correlated with the presence of erect glandular trichomes on the plants [5, 11, 21]. Although it was initially suggested that the viscosity of the exudate from the glandular trichomes determined larval mortality [13], it was later reported by Danielson et al. [4] that factors other than the sticky nature of the exudate may have contributed to larval mortality in resistant *Medicago* species. The same authors suggested that morphological and anatomical characteristics conditioned resistance to oviposition and that the role of the glandular trichomes on oviposi-

tion was uncertain [5]. In *Solanum berthaultii* the heads of glandular trichomes rupture upon insect contact discharging an exudate that rapidly undergoes oxidative polymerization and entraps insects [12]. PPO is the enzyme responsible for the oxidative polymerization and it constitutes up to 70 % of the protein content of the trichomes.

Up to now, it has not been demonstrated whether insect resistance in glandular-haired *Medicago* species is due to the mechanical entrapment of insects in a sticky substance exuded by glandular trichomes. Our results indicate that in *M. intertexta* the presence of glandular hairs is not associated with high values of PPO specific activity. Furthermore, while attempting to collect the exudate from individual trichomes on the glandular-haired species, we realised that their bulbous head did not rupture easily upon contact as in *S. berthaultii*. This suggests that the role of glandular trichomes in insect resistance is different in the two genera.

In order to characterise further the *Medicago* PPO, we studied the effect of DEDTC and 2,3-dimercaptopropanol (British Anti-Lewisite, BAL), which interact with the copper prosthetic group of PPO inhibiting the PPO activity [19]. Both BAL and DEDTC inhibited more efficiently the PPO of *M. sativa* than that of the other species at all the concentrations exploited (table II). Inhibition activity of BAL was markedly greater than DEDTC, in that BAL 0.1 mM lowered PPO activity by 63 % in

Table I. PPO enzymatic activity in stem tissue of *Medicago* species. The values are the mean ($\pm$ se) of three independent experiments.

<i>Medicago</i> spp.	Km (mM)	Specific activity units min ⁻¹ mg ⁻¹ proteins	
		Epidermis	Peeled stems
<i>M. sativa</i>	$6.2 (\pm 0.4) \times 10^{-1}$	0.24 ± 0.04	0.17 ± 0.08
<i>M. intertexta</i> ^g	$4.0 (\pm 0.6) \times 10^{-1}$	2.30 ± 0.08	0.54 ± 0.12
<i>M. intertexta</i> ^h	$5.7 (\pm 0.3) \times 10^{-1}$	1.55 ± 0.07	0.27 ± 0.06
<i>M. scutellata</i>	$5.7 (\pm 0.2) \times 10^{-1}$	0.73 ± 0.10	0.09 ± 0.04
<i>M. rugosa</i>	$3.7 (\pm 0.4) \times 10^{-1}$	1.47 ± 0.06	0.40 ± 0.04

^g Glabrous *M. intertexta*; ^h haired *M. intertexta*.

Table II. Effect of the inhibitors BAL and DEDTC on the PPO activity in stem epidermis of *Medicago* species.

<i>Medicago</i> spp.	BAL(mM)				DEDTC(mM)			
	0.1	0.5	1.0	3.0	0.1	0.5	1.0	3.0
<i>M. sativa</i>	37	24	18	15	100	57	41	34
<i>M. intertexta</i> ^g	86	47	40	33	100	72	61	55
<i>M. intertexta</i> ^h	50	41	38	23	81	71	60	49
<i>M. scutellata</i>	81	56	44	38	100	100	87	80
<i>M. rugosa</i>	78	69	54	40	100	100	100	63

^g Glabrous *M. intertexta*; ^h haired *M. intertexta*.

M. sativa and an average of 26 % in the other species, while the same concentration of DEDTC did not affect the enzymatic activity. Different results were reported in *Solanum berthaultii* where DEDTC was a more potent inhibitor than BAL [19]. These findings provide further evidence that i) PPO of susceptible *M. sativa* differs from that of resistant *Medicago* spp. and ii) *Medicago* PPOs differ from that contained in glandular trichomes of *S. berthaultii*.

PAGE analysis of PPO revealed two reproducible bands in all the *Medicago* species considered (figure 1). The M_r of the heavier PPO isoform was 180 kDa in *M. rugosa* and *M. scutellata*, 120 kDa in all the other species, while the M_r of the lighter PPO isoform was 75 kDa in *M. sativa* and 65 kDa in all the other spp. In figure 1 *M. scutellata* shows both 180 and 120 kDa bands but the 120 kDa band was not reproducible. It is worth noting that, at acrylamide concentrations higher than 7.5 %, the two PPO isoforms appeared as a doublet or triplet (data not shown). Plant PPOs have heterogeneous M_r values ranging from 30 to more than 200 kDa even if the usually reported molecular weights of PPO range from 30 to 72 kDa [16, 17, 22]. The nature of this variation remains unclear and it is suggested that PPO isoforms could be the result of a family of genes, oxidative aggregates, protease activity and alkylation by quinones [1, 17, 24]. Recently, a number of cDNAs and genes coding for plant PPO have been isolated and characterised [3, 9]. PPO expression in plants seems to be regulated by a complex gene family that in tomato is formed by up to seven

Figure 1. PPO staining of a 7.5 % polyacrylamide gel. The same volume (50 μ L) of proteins extracted from stem epidermis of different species were loaded. Lanes 1–5: *M. sativa*, haired *M. intertexta*, glabra *M. intertexta*, *M. rugosa*, *M. scutellata*.

nuclear genes characterised by different tissue-specific regulation [17]. In the genus *Nicotiana* the range reported for PPO isozymes was from 47 to 120 kDa [14]. It is possible that the 120 kDa PPO detected in this study is a dimeric form of the 65 kDa PPO, arising from association–dissociation mechanisms [16].

In conclusion, it is out of the question that, in the *Medicago* species here examined, PPO was more active in the epidermis than in other stem tissues and this fact, together with the natural defensive role of epidermis, and the great differences in specific activity detected between susceptible *M. sativa* and resistant *Medicago* spp., support the idea that PPO is involved in the resistance to biotic

stresses. However, qualitative differences were not detected between glabrous and glandular-haired *M. intertexta* for the PPO activity of the stem epidermis and, indeed glabrous *M. intertexta* had the highest value of PPO activity, indicating that at least for this species the anti-feeding effect of PPO may be due to the production of anti-quality factors. Further studies are necessary to screen for quantitative variation of epidermal PPO within the alfalfa germplasm to assess its correlation with resistance to biotic stresses. If this correlation exists then it will be possible to use epidermal PPO-quantitative-analysis as a marker for selecting resistant alfalfa genotypes.

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