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BRIEF COMMUNICATION

Activity of secreted cell wall-modifying enzymes and expression of peroxidase-encoding gene following germination of *Orobanche ramosa*

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Abstract

Radicle growth of germinated seed of the root parasite *O. ramosa* is shown to be rapidly accompanied by secretion of proteins including pectinolytic enzymes, polygalacturonase and rhamnogalacturonase. These secretions peaked between 4 to 8 d after induction of germination and remained constant for some further days in the case of polygalacturonases. After 6 d, germinated seeds secreted proteins which exhibit peroxidase activity. The latter may be correlated with expression of *OrPOX1*, a putative gene encoding for secreted peroxidase. The involvement of these enzymes in host root attack and haustorium formation by the parasite is discussed.

Additional key words: broomrape, cellulase, GR24, hemicellulase, parasitic plants, pectate lyase, stimulants of germination, virulence.

Orobanche ramosa L. is a root-parasitic flowering plant which causes important yield losses in rapeseed, tobacco and hemp in Europe (Benharrat *et al.* 2005). After a preconditioning step which requires adequate temperature and moisture conditions, seed germination is triggered by chemical signals released by the host roots (Riches and Parker 1995). Following germination, seedling radicle tip sticks to the host root and develops a haustorium which penetrates the host root cortex and connects vascular tissues. Broomrape penetrates host tissues by both mechanical action and enzymatic digestion and/or alteration of host cell walls by secreted enzymes including cellulase, hemicellulases, pectinolytic enzymes (PG, pectin methylesterase), peroxidase (POX) and proteases (Ben-Hod *et al.* 1993, Singh and Singh 1993, Sholmer-Ilan 1993, Antonova and Ter Borg 1996, Bar-Nun *et al.* 1996, Loesner-Goshen *et al.* 1998, Veronesi *et al.* 2005). These enzymes may fragilize host cell walls and particularly middle lamella, then facilitating progression of parasite intrusive cells in the host root.

The present study contributes to a better characterization of the enzymatic machinery of *O. ramosa* that is assumed to be involved in the infection process. Thus, a large number of cell wall-modifying enzymes were researched in the culture filtrates of germinated seeds, including cellulase, hemicellulases (arabinofuranosidase, xylosidase), pectinolytic enzymes [pectin methylesterase, pectate lyase, polygalacturonase (PG), rhamnogalacturonase (RG)] and peroxidase (POX). In addition, we isolated recently a cDNA encoding for a secreted POX (*orPOX1*, GenBank AY692263) from *O. ramosa*, some additional information about the pattern of *OrPOX1* expression following germination are also given.

Broomrape (*Orobanche ramosa* L.) seeds were collected from infested rapeseed fields in France (2003). Seeds were surface sterilized in sodium hypochlorite for 5 min, then rinsed twice with sterile distilled water. Fifty mg of seeds (around 10 000 seeds) were spread on a sterilized moistened fibre glass paper filter in a Petri dish. After preconditioning in darkness for 7 d at 25 °C, seeds were treated with 5 cm^3 of water (control) or 1 µg dm^{-3} GR24 (germination stimulant, Johnson *et al.* 1976) and incubated at 25 °C for two weeks. Each treatment was repeated simultaneously in 6 separate Petri dishes. Seed viability was estimated using Evan's blue at 1 g dm^{-3} from 3 sets of 200 preconditioned seeds per Petri dish.

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Abbreviations: PG - polygalacturonase; POX - peroxidase; RG - rhamnogalacturonase.
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Seed culture media, which contain excreted substances, were collected after increasing periods of treatment following preconditioning (0, 2, 4, 6, 8, 10, 12, 14 d), then filter sterilized (0.22 µm filter, *Millipore*, Saint Quentin en Yvelines, France) for enzyme assays (n = 6, data are means ± confidence intervals, P = 0.05, Student's t-test). Data are normally distributed and significant differences at P = 0.05 between culture filtrates were calculated using the Tuquey test (*SigmaStat*, *SPSS Inc.*, Chicago, USA).

Cellulase, hemicellulase, pectate lyase and pectin methylesterase activities were measured according to Bonnin *et al.* (1997). Polygalacturonase (PG) and rhamnogalacturonase (RG) activities were estimated by the release of reducing groups (Sturgeon 1990, Garg *et al.* 1999) from the polygalacturonic acid (PGA, *Sigma*, Saint Quentin Fallavier, France) and rhamnogalacturonan (in house prepared, Bonnin *et al.* 2001) substrates, respectively, following 10 min-incubation at 40 °C in a reaction medium containing 0.025 cm^3 of culture filtrate (around 0.5 to 5 µg proteins) and 0.225 cm^3 of 50 mM citrate buffer (pH 6.0) containing 0.1 g dm^{-3} PGA or 0.1 g dm^{-3} rhamnogalacturonan. The released reducing groups were measured spectrophotometrically (A_{600}) from 0.1 cm^3 of reaction medium. For both activities, D-galacturonic acid was used as a standard. POX activity was monitored (A_{465}) in a reaction medium (10 min, 35 °C) containing 0.025 cm^3 of culture filtrate (0.5 to 5 µg proteins) and 3 cm^3 of 100 mM acetate buffer (pH 4.7) containing 50 cm^3 dm^{-3} guaiacol, 100 mM H_2O_2 and 200 mM $CaCl_2$ (Honold and Stahmann 1968, Tahlil *et al.* 1999). Enzyme activities were calculated taking adequate controls into account. Total protein contents of culture filtrates were determined according to Bradford (1976).

In addition, the treated seeds were harvested for RNA extraction and RT-PCR analysis of *OrPOX1* expression,

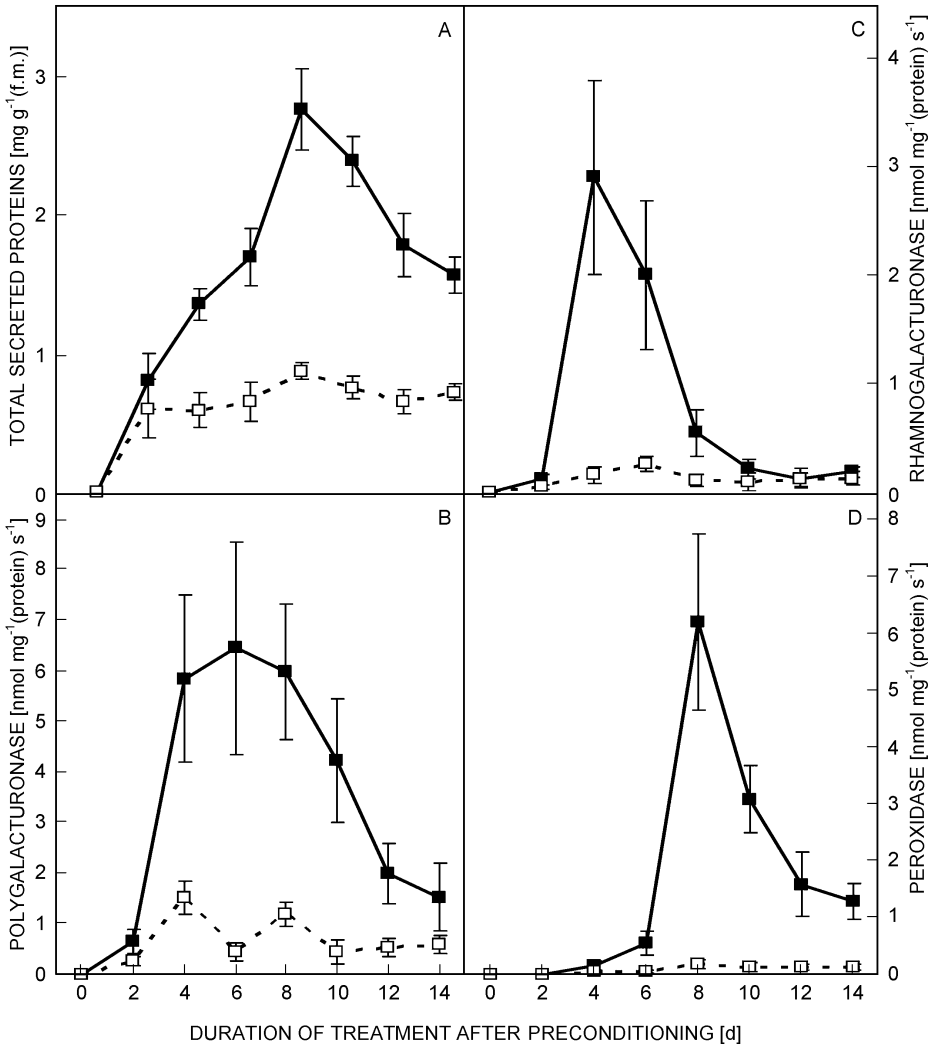

Fig. 1. Changes in total secreted protein content (*A*) and specific activity of PG (*B*), RG (*C*) and POX (*D*) in culture filtrates of water (*open squares*) and GR24-treated (*closed squares*) seeds of *O. ramosa* after preconditioning. Treatment was performed during 14 d from preconditioned seeds (day 0). Germination started by 2 d only for GR24-treated seeds. The seedlings were senescent from 12-d treatment. Data are means ± confidence intervals (n = 6, P = 0.05, Student's t-test).

as previously described by Delavault *et al.* (2002). Independent PCR reactions using equal aliquots (0.002 cm^3) of cDNA samples were performed using two *OrPOX1*-specific primers POX5 and POX11 (5'-TTTAGTGAAAAACAACAAATTTTCTGC, 5'-CATTTTCATGATTGCTTCGTTCAGGGATG).

Primer sets were chosen in such a way that a genomic cDNA amplification gave a product containing at least one intron, allowing a direct confirmation that RT-PCR products were of mRNA origin. Annealing conditions were empiricaly determined. Amplification of the *18S* gene exhibiting constitutive expression was used as a positive control. Data correspond to one experiment among three independent experiments.

Following preconditioning, seed viability percentage was estimated at around 90 %. When constantly treated with water following preconditioning, no seed germinated. In contrast, synchronized germination was observed in the presence of GR24. Following GR24-induced germination, viability percentage for the germinated seeds was constantly high (around 90 %) up to 10 d of culture in presence of GR24, then rapidly decreased and reached the low value of 50 % at 14 d of culture. Furthermore, the surviving germinated seeds had lost their ability to infect host roots. For shorter culture times, total protein leakage from germinated seeds increased gradually with age up to 8 - 10 d of culture (Fig. 1*A*). Then protein contents in culture filtrates decreased significantly. This may be related to senescence. Indeed, further development of broomrape is fully host-dependent and needs attachment to the host root. Except at the second day of treatment following preconditioning, the extent of protein excretion from the water-treated seeds was significantly lower at any time of culture and remained constant with time.

Among all the enzymes tested in the culture filtrates of water- and GR24-treated seeds, only PG, RG and POX activities were successfully detected (positive controls were successfully performed with commercial enzymes as PG and RG from *Aspergillus niger* and horseradish POX). Very low activities were detected at any time of culture for all three enzyme PG, RG and POX in the culture filtrates of constantly water-treated seeds (Fig. 1*B*,*C*,*D*). In contrast, the specific activity was clearly increased for the pectinolytic enzymes PG and RG in the culture filtrates of germinated seeds that were collected 4 d after GR24 addition (Fig. 1*B*,*C*). Maximal specific activity was maintained for PG up to 10 d (activities were not significantly different between 4 and 10 d), and the decrease that was observed following longer times of culture could be related to senescence. In contrast, RG activity decreased significantly from the eighth day of culture when high seed viability was still maintained. The pattern of POX activity, which peaked at 8 d of culture (Fig. 1*D*), differed from those of PG and RG. Nevertheless, the reached activity was close to the maximal activities of PG and RG. A significant decrease in POX activity was detected from the tenth day of culture suggesting that it is transitory.

The full length *OrPOX1* cDNA was preliminary sequenced and the deduced peptide sequence matched with secreted POX proteins (data not shown). The preconditioned seeds displayed low amounts of *OrPOX1* transcripts when constantly treated with water (Fig. 2). Similarly, abundance of *OrPOX1* transcripts was low in the germinated seeds at the second day of treatment with GR24 (Fig. 2). On the other hand, following 4 to 10 d of culture the germinated seeds accumulated *OrPOX1* transcripts at a relatively stable rate during this long period. The further low *OrPOX1* expression is assumed to be related to senescence. Though the transitory POX activity that was detected in the culture filtrates of the germinated seeds after 8 d of culture may result from any POX proteins in addition to POX1, *OrPOX1* gene expression is shown to be rapidly induced following GR24-induced germination, assuming that *OrPOX1* contributed to the detected POX activity.

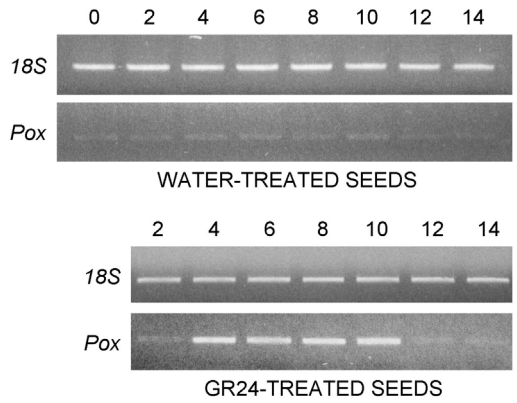

Fig. 2. Changes in accumulation of *OrPOX1* (*Pox*) transcripts in the water- and GR24-treated seeds of *O. ramosa*. Treatment was performed during 14 d from preconditioned seeds (day 0). Germination started by 2 d only for GR24-treated seeds. The seedlings were senescent from 12-d treatment. The *18S* gene exhibiting constitutive expression was used as a positive control of RT-PCR reaction.

In conclusion, the two active PG and RG proteins were shown to be rapidly secreted by *Orobanche ramosa* following germination. Loesner-Goshen *et al.* (1998) reported the evidence of a pectin methylesterase (another pectinolytic enzyme) at the tomato/*O. aegytiaca* interface, giving the first insights for the involvement of pectinolytic enzymes in the invasion by *Orobanche*. Furthermore, activities of two pectinolytic enzymes (PG and PME) have been shown to be linked to virulence of *O. cumana* parasitazing sunflower (Veronesi *et al.* 2005). Concerning POX, such enzymes may act on the host root surface as a laccase or a suberinase in degrading lignin or suberin as it was shown for POX secreted by ligninolytic fungi (Johansson and Nyman 1996, Cullen 1997), or as a H_2O_2-dependent phenol oxidase modifying specific host quinone compounds into a signal molecule that induces haustorium differentiation as it was demonstrated for the secreted POX of the parasitic plant

Striga asiatica (Kim *et al.* 1998). Beside these findings, Antonova and Ter Borg (1996) reported in contrary that high virulence of another *Orobanche* species *O. cumana* against sunflower is not correlated with high secretion of POX. Secretion of an active POX enzyme that is supposed to be related to *OrPOX1* gene expression is obviously shown in the present study for the germinated seeds of *O. ramosa*. Nevertheless, clarification of the role of the secreted POX in *Orobanche* virulence needs further investigations.

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