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Changes in the activity of the alternative oxidase in *Orobanche* seeds during conditioning and their possible physiological function

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

The appearance of the activity of the cyanide insensitive, alternative oxidase (AOX), pathway of oxygen uptake was followed in seeds of *Orobanche aegyptiaca* during conditioning. The pathway becomes operative during conditioning, up to day three as determined by inhibition of oxygen uptake of the seeds by propyl gallate. At the same time an increasing percentage of oxygen uptake is insensitive to cyanide and an increased oxygen uptake, responsive to propyl gallate, is induced by brief salicylic acid treatment of seeds. By day six of conditioning, these responses decrease and the AOX pathway could not be detected in germinating seeds, after treatment with a germination stimulant. These results were confirmed by following the reaction of extracts of fractions enriched with mitochondria from the conditioned seeds, using a specific antibody against AOX. Treatment of the seeds with inhibitors of AOX during conditioning significantly inhibited their subsequent germination. Addition of hydrogen peroxide after 4 and 7 days of conditioning resulted in reduced germination. In addition treatment of seed with propyl or octyl gallate during conditioning reduced the infection of tomato plants by *Orobanche* seeds and the development of tubercles of the parasite on the host roots. These results together indicate that the operation of AOX during conditioning has a significant function on the subsequent germination behaviour and pathogenicity of the root parasite. Some potential practical applications of these findings are discussed.

Keywords: *Orobanche aegyptiaca*; Orobanchaceae; Conditioning; Germination; Alternative oxidase pathway; AOX protein; Oxygen uptake; Inhibition of AOX; H_2O_2; Reactive oxygen species

1. Introduction

The germination of seeds of parasitic plants such as *Orobanche* spp. are characterized by two phases, a phase of conditioning (sometimes referred to as preconditioning) during which the seeds take up water and show active metabolism, but are unable to respond to germination stimulants, and a germination phase. This second phase begins after the termination of conditioning (Joel et al., 1989). The sharp transition in *Orobanche* seeds between these two phases appears to be unique for *Orobanche* and similar root parasites like *Striga* spp. This germination behaviour is described in detail by Joel et al. (1995).

Previous work (Bar Nun and Mayer, 1993; Mayer and Bar-Nun, 1994, 1997) indicated that part of the oxygen uptake during conditioning of *Orobanche aegyptiaca* seeds was mediated through the cyanide resistant pathway, often called the 'alternative oxidase pathway' (AOX). Evidence for this included the failure of cyanide to inhibit part of the oxygen uptake, the partial inhibition of oxygen uptake by salicylhydroxamic acid (SHAM) and the induction of increased oxygen uptake by salicylic acid (SA). The activity of the cyanide resistant respiration pathway has been reported in some germination stages of many plant species (Mayer and Poljakoff-Mayber, 1989). This pathway can be induced by salicylic acid (Kapulnik et al., 1992; Raskin, 1992; Vanlerberghe and McIntosh, 1997), although salicylic acid may have additional effects (Xie and Chen, 1999).

The function of the cyanide resistant pathway in plant tissues is still not resolved (Simons and Lambers, 1999). Recent reports suggest that a potential function of the AOX is in defence mechanisms of plants, e.g. against

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oxidative stress, possibly by lowering the level of reactive oxygen species in plant mitochondria (Maxwell et al., 1999). Hence it is tempting to invoke a similar function of AOX during the conditioning of *Orobanche* seeds. It is at least plausible that while conditioning (but not germinating) the seeds are exposed to stress, possibly due to reactive oxygen species such as hydrogen peroxide. At the same time hydrogen peroxide, which is toxic to plant tissues can ameliorate germination of some seeds (Ogawa and Iwabuchi, 2001) and the formation of hydrogen peroxide during the germination of radish seeds has been shown (Schopfer et al., 2001). Peroxidase activity has been shown to develop in the micropylar region of the endosperm of imbibed tomato seeds and the possible function of hydrogen peroxide prior to radicle protusion has been discussed (Morohashi, 2002).

It should be possible to obtain some insight into the function of AOX during conditioning of *Orobanche* by determining the level of its activity, and following the level of the enzyme and the response of respiration to inhibitors of AOX. Previous evidence was based partly on the use of SHAM. However, this inhibitor is not sufficiently specific for AOX, and therefore additional inhibitors should be used, for example propyl gallate (PG). At the same time studying the effect of AOX inhibitors, applied during conditioning or germination, on germination of the seeds should also give some indication of the validity of this hypothesis.

The parasitic weed *Orobanche* spp. is a serious scourge in agriculture. It causes severe damage in many important crops and so far there are only limited ways to control it. Germinated seeds adhere to host root, and develop a haustorium that penetrates the root, deriving water and nutrients from it. It was recently suggested that the ability to interfere in seed germination may alleviate *Orobanche* damage in the field (Joel, 2000). In the following we report not only on the effect of inhibitors of AOX on respiration, AOX activity and on AOX induction by salicylic acid during conditioning, but also on the effect of these inhibitors on the subsequent germination. In addition, we examined the hypothesis that the application of AOX inhibitors during seed conditioning would reduce the extent of host plant infection by the parasite.

2. Results and discussion

2.1. Oxygen uptake

Measurements of the respiration rate of two seed lots were made. The results of the measurements on the 1999 seed lot are recorded in Table 1. The respiration rate of the old seed lot was significantly lower despite the fact that these seeds still germinated fully in response to GR 24 (a germination stimulant, results not shown).

Oxygen uptake of the seeds responded to salicylic acid, although the response was variable and differed between the two seed lots. In both lots respiration began to decline after 6 days of conditioning. Oxygen uptake of conditioned seeds was partially inhibited by propyl gallate, indicating that at least a part of the oxygen consumption was mediated by the alternative oxygen uptake pathway. Inhibition varied from 33% on day three to 17–23% on days 4–6 in the 1999 seed lot (Table 1). Similar values of inhibition were noted in the older seed lot (results not shown). The old seed lot also responded to SA even after 5 and 6 days (results not shown). However, in the new seed lot, the response to SA was absent after 6 days of conditioning. The response to PG on days 5 and 6 was slightly lower than on days 3 and 4. The results of the combined treatment with SA followed by PG in the 1999 seed lot, resulted in reduction of oxygen uptake below that of PG alone. This could be interpreted as showing that a switching from normal, cytochrome mediated pathway to the cyanide resistant oxygen uptake pathway took place and that the latter did not become engaged again when the AOX was inhibited, but possible toxic effects of the combined treatment cannot be ruled out. This reduction was also observed when the reverse treatment, PG followed by SA, was used (Table 1). The experimental data showed some variability and many replicates were needed to obtain reliable results. Every experiment was therefore repeated in at least three to five independent experiments.

OG was active at much lower concentrations than PG (Table 1). This is in accord with the results of Hoefnagel et al. (1995), who also showed that octyl gallate was more active than propyl gallate. Treatment with SA followed by OG reduced oxygen uptake somewhat more than treatment in the reverse order, and this was also the case for PG followed by SA. The reason for this effect is not obvious at the moment. It is clear that part of the oxygen uptake is mediated via the pathway of the alternative oxygen uptake mechanism, which is known to respond to propyl gallate and to octyl gallate (Moore and Siedow, 1991; Hoefnagel et al., 1995; Wagner and Moore, 1997).

The effect of cyanide on the oxygen uptake of the seeds was studied at two cyanide concentrations, 1 mM and 2 mM (Table 2). The higher KCN concentration inhibited oxygen uptake by 58% on day 3 and by 40% on day 6. After SA treatment, KCN (2 mM) inhibited oxygen uptake by 53% on day 3 and 80% on day 6. Thus in all cases a substantial part of the oxygen uptake was mediated via a cyanide sensitive pathway.

2.2. Immunodetection of the alternative oxidase

The protein pattern in extracts from *Orobanche* have previously been described only for total protein (Bar-Nun and Mayer, 1993). In our present research extracts

Table 1
Rate of oxygen uptake by *Orobanche aegyptiaca* seeds (seed lot 1999) after conditioning for various periods of time, following exposure to salicylic acid or propyl gallate or octyl gallate combinations of SA and PG or SA and OG

Conditioning period	Oxygen uptake μl O_2^{-1}.100 mg^{-1} initial dry seeds							
	H_2O	H_2O + SA	H_2O + PG	H_2O + SA then PG	H_2O + PG then SA	H_2O + OG	H_2O + SA then OG	H_2O + OG then SA
3 days	2.03±0.09	3.24±0.46	1.37±0.25	1.16±0.09	1.47±0.12	1.6±0.03	1.2±0.05	1.9±0.22
4 days	2.60±0.52	3.45±0.37	2.15±0.32	1.80±0.30	2.00±0.03			
5 days	2.42±0.20	3.10±0.11	2.10±0.13	1.60±0.22	2.05±0.2			
6 days	1.34±0.11	1.36±0.33	1.03±0.19	0.98±0.06	1.14±0.26	1.2±0.19	0.93±0.05	1.07±0.05

Results as μl O_2^{-1} 100 mg^{-1} initial dry weight of seeds. min^{-1} ±S.D. SA = salicylic acid; PG = propyl gallate; OG = octyl gallate. Salicylic acid treatment, 20 μM for 30 min at room temperature. *n*-Propyl gallate treatment, 0.4mM for 30 min at room temperature. Combined treatment for 30 min each with SA and then PG or PG followed by SA. *n*-Octyl gallate treatment, 50 μM for 30 min at room temperature. Octyl gallate (1.4 mg) was dissolved in 0.5 ml DMSO containing 10 mM HCl and then diluted with 100 ml water. Combined treatment for 30 min each with SA and then OG or OG followed by SA.

Table 2
Response of oxygen uptake by *Orobanche aegyptiaca* seeds to cyanide, after conditioning for various periods of time, following exposure to salicylic acid or propyl gallate or both

Co.	H_2O	H_2O + CN	H_2O + SA	H_2O + SA then CN	H_2O + PG	H_2O + PG then 2 mM CN	H_2O + SA then PG	H_2O + SA then PG +2 mM CN	H_2O + PG then SA
3	2.0	1.1	3.2	1.6	1.4	0.27	1.15	0.04	1.47
4	2.6	–	3.45	–	2.1	–	1.8	–	2.0
5	2.4	–	3.1	–	2.1	–	1.6	–	2.0
6	1.3	0.9	1.4	0.66	1.0	0.03	0.98	0	1.1

Results as μl O_2^{-1} 100 mg^{-1} initial dry weight of seeds. min^{-1} ±S.D. Co = length of conditioning period, days; PG = 0.4 mM propyl gallate; SA = 20 μM salicylic acid; CN 1 mM KCN (unless otherwise indicated). Treatment with SA and PG for 30 min; treatment with KCN for 40 min. Seed lot 1999.

of the fractions enriched with mitochondria were examined at different times during conditioning. The pattern of electrophoresis and the Western blots are shown in Fig 1A and B. In the mitochondrial extracts protein bands between 40 and 66 kDa first become increasingly marked and then weaken, while low molecular bands appear particularly after GR 24 treatment (Fig.1A, 8d.). The molecular weight of the bands reacting with the antibody was calculated from that of the molecular weight markers. The Western blots showed a distinct but weak band for AOX at 32 kDa in extracts of dry seeds. After 1 and 2 days of conditioning this band was also present, somewhat more intense. After 3 days of conditioning this band becomes much more marked. However, no corresponding band could be detected after 6 days of conditioning or in GR24 treated seeds 8 days after imbibition. No AOX could be immunodetected even after overloading the gels (by a factor of 3) with seed extracts from day 6. Since the AOX activity responds to short treatment with salicylic acid, we also examined the Western blots after such treatment. In the dry seeds, the pattern without and with salicylic acid was the same. After 2 and 3 days, salicylic acid treatments resulted in slightly more intense bands of AOX. This increase in intensity is more marked after 3 days. More strikingly, extracts from 3-day old seeds that were treated with salicylic acid showed a new band, at about 20 kD, reacting with the antibody against AOX. This may indicate the partial degradation of the protein of AOX, which has been reported previously (Elthon et al., 1989), although it is possible that this 20 kD band was not detected in the other stages, due to the lower apparent amount of AOX detected in them. It is at this time that the oxygen uptake of the seeds responds most markedly to salicylic acid treatment. After 6 days conditioning, when there is hardly any response in oxygen uptake to salicylic acid treatment (Tables 1 and 2), the salicylic acid treatment leads to the appearance of a band reacting with the antibody. The electrophoretic pattern of the extracts changed only marginally after treatment of the seeds with salicylic acid (results not shown).

These changes in the immunoreaction with and without salicylic acid parallel the response of the conditioned seeds to salicylic acid, and to the apparent levels of AOX mediated oxygen uptake. They are consistent with the idea that the AOX activity in the imbibed seeds is regulated by structural changes in the protein including aggregation or de-aggregation. The nature of the band at about 20 kDa, which was only detected in seeds conditioned in 3 days is not clear at present. During the transition from the conditioned state to the state at

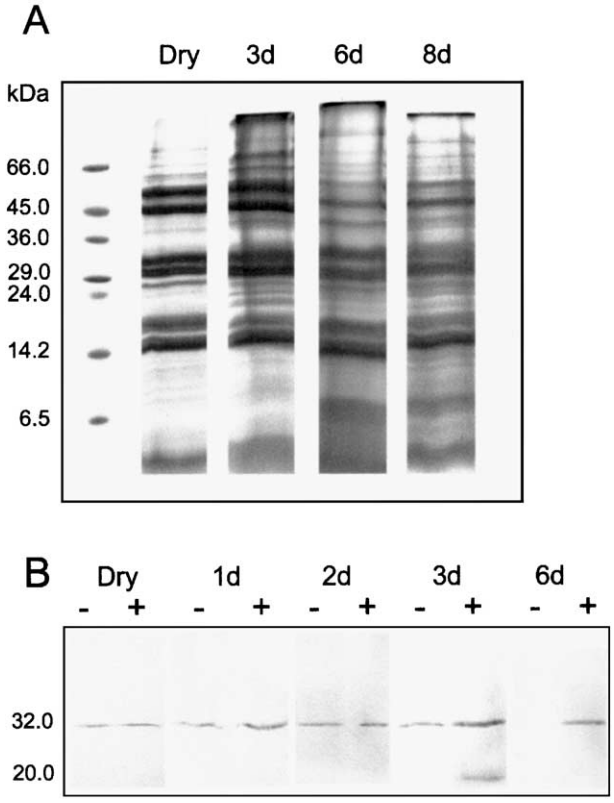

Fig. 1. Electrophoretic separation by SDS–PAGE and immunoblotting of proteins in extracts of a fraction enriched with mitochondria of *Orobanche aegyptiaca* during conditioning and germination. (A) Gels stained for protein with Coomassie Blue R 250. The lanes represent extracts from dry seeds and seeds conditioned for 3 or 6 days (3d, 6d) and seeds treated after 6 days of conditioning with GR24 for 2 days (8d). (B) Immunoblot of gel after Western blotting using monoclonal antibody against AOX. Dry: extract from dry seeds; 1d, 2d, 3d, and 6d extracts from seeds conditioned for 2, 3 or 6 days respectively. (−) = Extracts from fraction enriched with mitochondria of seeds imbibed in water. (+) = Extracts from fraction enriched with mitochondria of seeds treated for 30 min with salicylic acid before preparation of mitochondria enriched fraction and extraction for immuno-blotting. Left hand lane in A and B: molecular weight markers, (Sigma 3913 low molecular weight markers, 6500–66,000 Da).

which the seeds respond to germination stimulants, the protein for AOX can no longer be detected using the specific antibody. Whether this is as a result of turnover, structural changes or controlled breakdown is not clear.

2.3. Effect of AOX inhibitors and of hydrogen peroxide on germination

Treatment of seeds with salicylic acid during conditioning, at concentrations between 10 and 200 μM, had no significant effect by itself on germination. Thus an increased activity of AOX did not apparently affect the final germination steps of the seeds. However, the application of hydrogen peroxide to the seeds at different times during the conditioning period or after application of the germination stimulant (i.e. at the very beginning of germination) did show a very significant effect on the final germination percentage (Table 3). Early application increased the germination percentage, while its application after conditioning reduced it.

Table 3
Germination of *Orobanche* seeds following exposure to hydrogen peroxide at various times: at start of conditioning (day 0), in the middle of conditioning (day 4) and after conditioning, during germination stimulation (day 7)

Day of treatment	% Germination	
	1 mM H_2O_2	5 mM H_2O_2
Day 0	76.7±4.0	70.3±0.5
Day 4	40.3±2.0	42.7±9.8
Day 7	33.0±2.4	28.0±1.6
Control	59.0±3.5	

Results are given as germination percentage ±S.E.

Table 4
Germination of three species of *Orobanche* seeds following exposure, during conditioning, to different concentrations of propyl gallate (PG), or octyl gallate (OG)

Inihbitor used		Germination		
		O aegyptiaca	*O. crenata*	*O cumana*
Propyl gallate	50 μM	97.3±0.3	98.3±0.6	98.2±2.6
	100 μM	98.1±1.4	82.0±1.9	88.3±8.0
	400 μM	77.2±1.1	72.7±1.5	36.8±2.1
Octyl gallate	50 μM	99.1±0.8	83.2±0.8	97.9±0.5
	100 μM	96.7±1.0	83.9±0.7	79.8±3.1
	400 μM	54.8±0.6	55.5±2.7	3.2±0.3

Results as percentage germination of the control ±S.D. The derivatives of gallic acid were first dissolved in DMSO, containing 10 mM HCl and then diluted with water. The final DMSO concentration was 0.5%, which was not toxic to the seeds.

Treatment of the seeds at different stages of conditioning with inhibitors of AOX had a clear-cut effect on germination of *O. aegyptiaca*. Propyl gallate and octyl gallate reduced germination percentage appreciably (Table 4), which positively corresponds to our results with oxygen uptake in this species. An even stronger inhibition was observed in two additional species (*O. crenata* and *O. cumana*), which belong to another section within the genus *Orobanche*. In all species octyl gallate was active at lower concentrations than propyl gallate. The effect of hydrogen peroxide and the influence of AOX inhibitors on germination together support the idea that repression of AOX activity during conditioning can result in an increase in the level of reactive oxygen species in the seeds, which apparently has an adverse effect on subsequent germination.

2.4. Effect of AOX inhibitors on infection of host roots

Tomato plants, which were exposed to *Orobanche aegyptiaca* seeds that were conditioned in the presence

of AOX inhibitors, showed a significantly reduced degree of infection (Table 5). While propyl gallate at 0.4 mM reduced infection by more than 50%, octyl gallate was even more effective, reducing infection by 65% at 0.4 mM, and almost 45% at a lower concentration of 0.1 mM. These results are consistent with the germination results given above, supporting the assumption that lower rates of infection are due to reduced germination rates.

3. Concluding remarks

The results clearly suggest that the AOX pathway does play an important role during the initial stages of conditioning of *Orobanche* seeds. Although the physiological function of this pathway is still unclear, a possible function might be the ability, during its operation, to remove active oxygen species (Wagner and Moore, 1997) that could arise during conditioning and cause damage to the seed. Apparently the AOX studied here belongs to the induced type of this oxidase, as discussed by Considine et al. (2002). Indeed, a late application of hydrogen peroxide to the seeds reduced germination percentage, which is consistent with such seed damage. We assumed that by inhibiting AOX, the removal of active oxygen species in the seed would slow down leading to a reduced germination percentage, resulting in lower rates of host infection by the parasite. Our results support this hypothesis, showing a significant drop in the germinability of seeds treated with propyl gallate or octyl gallate during conditioning, and a consequent decline in infection of tomato plants by *O. aegyptiaca*.

These findings have twofold significance: First, they suggest the possible use of AOX inhibitors as specific agents for reducing the damage caused by parasitic weeds in the field. The studies of metabolic events during conditioning should be extended in order to use this strategy for the control of parasitic weeds. In addition, the study shows that basic understanding of specific metabolic pathways in parasitic weeds can lead to the development of new means for its management.

Table 5
Mean number of *O. aegyptiaca* infections on the root system of tomato plants, following exposure of the seeds of *Orobanche aegyptiaca* during conditioning, to propyl gallate (PG) or octyl gallate (OG)

	Infections per tomato plant
Control	50.2±18.1
0.4 mM PG	22.5±4.9
0.1 mM OG	28.6±4.7
0.4 mM OG	17.8±4.1

The seeds were treated with salicylic acid prior to the gallate treatments. Control plants were exposed to seeds that were conditioned in water without SA and gallate treatments. The experiment was done in polyethylene bags, two plants per bag, five bags per treatment.

4. Experimental

4.1. Oxygen uptake

Seeds of *Orobanche* harvested in 1993 and 1999 at Kibbutz Usha were used for the experiments on respiration as determined by oxygen uptake. Both seed lots gave 80–100% germination after conditioning for six days followed by exposure to 5 ppm of the synthetic strigol analogue GR-24 (Parker and Riches, 1993). However the old seed lot had a lower rate of oxygen uptake and had a poorer response to 20 μM salicylic acid (SA).

Seeds were conditioned in water after being surface-disinfected as described by Bar-Nun and Mayer (1993). After various periods of time the seeds were weighed and oxygen uptake measured using a Clark type oxygen electrode with a ten-fold increase in sensitivity (Lozano and Mayer, 1990). The stability of the system was improved by placing the water bath containing the cells for measurement and the oxygen electrode in a Faraday cage. The stirrer was gold plated to avoid artifacts due to oxidation of the metal of the stirrer.

Oxygen uptake of seeds was measured after conditioning or after treating the conditioned seeds for 30 min with 20 μM salicylic acid or with 0.4 mM propyl gallate or 50 μM octyl gallate either immediately before measurement or following exposure to salicylic acid.

4.2. Preparation of extracts for immunodetection of AOX

Seeds of *Orobanche aegyptiaca* (collected at Mey-Ami, Israel in 2000) were conditioned and germinated at 24 °C as previously described (Bar-Nun and Mayer, 1993). In order to prepare material for electrophoresis and Western blotting a fraction enriched with mitochondria was isolated essentially as described by Marbach and Mayer (1976), with some modification. Dry (2 g) or imbibed seeds (4 g) were ground in phosphate buffer (0.05 M, pH 7.2) containing 0.4 M sucrose, 1 mM EDTA and 0.05 M cysteine. The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was then centrifuged at 20,000 *g* for 20 min and the pellet resuspended in 400 μl sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol and 10% mercaptoethanol). The samples were treated at 90 °C for 3 min.

4.3. Electrophoresis

Samples of extracts of fractions enriched with mitochondria containing 60–80 μg protein were loaded on to

SDS acrylamide gels (4% stacking, 12.5% resolving gel) and electrophoresis carried out as described by Laemmli (1970). Gels were run for 45 min at constant voltage (100 V) and then for a further 105 min at 150 v. The gels were stained with Coomassie Blue R 250. The gels were run together with low molecular weight markers (65,400–66,000 Da, Sigma 3913).

4.4. Western blotting and immunodetection

The protein of AOX was detected using a monoclonal antibody diluted 1:100, obtained from Professor Thomas E. Elthon. We tested the antibody using extracts from mango as described by Considine et al. (2001). Two AOX bands between 33 and 36 kDa were detected as described by Considine et al. (2001), although the exact molecular weight differed slightly from that reported by these authors. This confirms the validity of the methodology.

Proteins were transferred from the gels to nitrocellulose (Schleicher and Schuell, 0.45 μm pore size) using a transblot system with Tris 25 mM-glycine192 mM dissolved in MeOH pH 8.3 as a standard buffer. Transfer was for 2 h at 450 mA. Nonspecific binding sites on the blots were blocked by shaking for 1 h with 3% non-fat dry milk powder (Sigma) dissolved in TBST (Tris 0.05 M, pH 7.5, containing 0.138 M NaCl, 0.0027 M KCl and 0.05% Tween 20).

After incubation for 1 h the blots were washed four times with TBST buffer and then incubated for 1 h with anti-mouse IgG (whole molecule) alkaline phosphatase conjugate diluted 1:5000 (Sigma) as the secondary antibody. After four further rinses the blots were developed in the dark using 5-bromo-4-chloro-3- indolyl phosphate/nitro tetrazolium blue liquid (Sigma).

4.5. Protein determination

For protein determination extracts were dialysed for 48 h, with several changes, against distilled water to remove SDS and other interfering compounds. Protein was determined using the method of Shakir et al. (1994).

4.6. Germination in the presence of AOX inhibitors and of hydrogen peroxide

Seeds were surface sterilized by soaking for 2 min in 70% EtOH, then 10 min in 1% NaOCl (with 0.05% Tween 20), washed in sterile water and allowed to dry. About 300 seeds were then uniformly scattered on each 4 cm disc of Whatman GFA filter paper that was presoaked with sterile water and placed in a Petri dish. The seeds were allowed to condition for 6 days at 22–25 °C (*O. aegyptiaca* and *O. cumana*), or 18–20 °C (*O. crenata*). On day 7 the medium was blotted, and the synthetic germination stimulant GR24 was applied at 5 ppm for seeds of *O. aegyptiaca,* 10 ppm for *O. crenata,* and 20 ppm for *O. cumana*. The treated seeds were kept in the Petri dish under similar conditions for additional 7 days to allow germination. Germination was recorded under a Zeiss SV8 dissecting microscope.

4.7. Seed treatment during conditioning

Hydrogen peroxide treatment (1 or 5 mM) was given on day 0, 4, or 7, after blotting the conditioning solution from the paper discs. The AOX inhibitors propyl gallate (PG), and octyl gallate (OG) were used at different concentrations in the conditioning medium instead of water. In these experiments the solution was changed once a day. In case of a combined treatment, SA was given 3 h prior to the application of the inhibitor. All experiments were done at least twice, in 3 replicates.

4.8. Host infection in the presence of AOX inhibitors

Dry seeds of *O. aegyptiaca,* treated as above, were evenly spread on a wet 28×11.5 cm sheet of GFA filter paper in a polyethylene bag (Linke et al., 2002), and kept in a growth cabinet at 22–25 °C. SA (10 ml, 20 μM) was added 3 days later, and on each of the next 5 days the conditioning solution was changed with a fresh solution of SA (20μM) together with either propyl gallate (400 μM) or octyl gallate (100–400 μM). Then young tomato plants were planted in the bag, allowing their root systems to develop on the surface of the GFA filter paper, adjacent to the treated broomrape seeds. No germination stimulant was added as the seeds were stimulated by the tomato root exudates. One month later the number of *Orobanche* tubercles that developed on tomato roots was counted. Conditioning in water without SA and inhibitors served as controls. The experiment was conducted with five replicates.

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References

Bar Nun, N., Mayer, A.M., 1993. Preconditioning and germination *of Orobanche* seeds: respiration and protein synthesis. Phytochemistry 34, 39–45.

Considine, M.J., Daley, D.O., Whelan, J., 2001. The expression of alternative oxidase and uncoupling protein during fruit ripening of mango. Plant Physiol. 126, 1619–1629.

Considine, M.J., Holtzapffel, R.C., Day, D.D., Whelan, J., Millar, A.H., 2002. Molecular distinction between alternative oxidase from monocots and dicots. Plant Physiol. 129, 949–953.

Elthon, T.E., Nickels, R.L., McIntosh, L., 1989. Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. Plant Physiol. 89, 1311–1317.

Hoefnagel, M.H.N., Wiskitch, J.T, Madgwick, S.A., Patterson, Z., Oettmeier, W., Rich, P.R., 1995. New inhibitors of the ubiquinol oxidase of higher plant mitochondria. Eur. J. Biochem. 233, 531–537.

Joel, D.M., Back, A., Kleifeld, Y., Gepstein, S., 1989. Seed conditioning and its role in *Orobanche* seed germination: inhibition by paclobutrazol. In: Wegmann, K., Musselman, L.J. (Eds.), Progress in Orobanche Research. Proc. International Workshop on Orobanche Research. Obermarchtal, FRG, pp. 147–156.

Joel, D.M., Steffens, J.C., Matthews, D.E., 1995. Germination of weedy root parasites. In: Kigel, J., Galili, G. (Eds.), Seed Development and germination. Marcel Dekker Inc, New York, pp. 567–598.

Joel, D.M., 2000. The long-term approach to parasitic weeds control: manipulation of specific developmental mechanisms of the parasite. Crop Protection 19, 753–758.

Kapulnik, Y., Yalpami, N., Raskin, I., 1992. Salicylic acid induces cyanide resistant respiration in tobacco cells suspension culture. Plant Physiol. 100, 1921–1926.

Laemmli, U.K., 1970. Cleavage of the structural proteins during the assembly of the head of the bacteriophage T4. Nature 227, 680–685.

Linke, K.-H., Joel, D.M., Kroschel, J., 2002. Observation of the underground development. In: Kroschel, J. (Ed.), Manual of Parasitic Weed Research and Extension. Kluwer Academic Publishers b.v., pp. 53–59.

Lozano, J.L., Mayer, A.M., 1990. Water relations and oxygen uptake by two lines of corn differing in storage performance. Israel J. Bot. 39, 347–354.

Marbach, I., Mayer, A.M., 1976. Respiration and utilization of storage materials in wild and cultivated pea seeds during germination. Physiol. Plant. 38, 126–130.

Maxwell, D.P., Wang, Y., McIntosh, L., 1999. The alternative oxidase lowers mitochondrial reactive oxygen production by plant cells. Proc. Nat. Acad. Sci. USA 96, 8271–8276.

Mayer, A.M., Bar Nun, N., 1994. Metabolism during preconditioning and germination of *Orobanche aegyptiaca*. In: Pieterse, A.H., Verkleij, J.A.C., ter Borg, S.C. (Eds.), Biology and Management of *Orobanche*. Proc. 3rd International Workshop on *Orobanche* and related *Striga* Research. Royal Tropical Institute Amsterdam, pp. 146–156.

Mayer, A.M., Bar Nun, N., 1997. Germination of *Orobanche* seeds: some aspects of metabolism during preconditioning. In: Ellis, R.H., Black, M., Murdoch, A.J., Hong, T.D.S. (Eds.), Basic and Applied Aspects of Seed Biology. Kluwer Academic, Dordrecht, pp. 633–638.

Mayer, A.M., Poljakoff-Mayber, A., 1989. The Germination of Seeds, fourth ed. Pergamon Press, Oxford.

Moore, A.L., Siedow, J.N., 1991. The regulation and nature of the cyanide resistant alternative oxidase of plant mitochondria. Biochim. Biophys. Acta 1059, 121–140.

Morohashi, Y., 2002. Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protusion. J. Experimental Botany 53, 1643–1650.

Ogawa, K., Iwabuchi, M., 2001. A mechanism for promoting the germination of *Zinnia elegans* seeds by hydrogen peroxide. Plant Cell Physiol. 42, 286–291.

Parker, C., Riches, C.R., 1993. Parasitic Plants of the World: Biology and Control. CAB International, Oxford.

Raskin, I., 1992. Role of salicylic acid in plants. Ann. Rev. Plant Physiol. Mol. Biol. 43, 439–463.

Shakir, F.K., Audilek, D., Drake III, A.J., Shakir, K.M.M., 1994. A rapid protein determination by modification of the Lowry procedure. Analytical Biochem. 216, 232–233.

Schopfer, P., Plachy, C., Frahry, G., 2001. Release of reactive oxygen intermediates (superoxide radicals,hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiol. 125, 1592–1602.

Simons, B.H., Lambers, H., 1999. The alternative oxidase: is it a respiratory pathway allowing plants to cope with stress? In: Lerner, H.R. (Ed.), Plant Responses to Environmental Stresses. Marcel Dekker, New York, pp. 265–286.

Vanlerberghe, G.C., McIntosh, L., 1997. Alternative oxidase: from gene to function. Ann. Rev. Plant Physiol. Mol. Biol. 48, 703–734.

Wagner, A.M., Moore, A.L., 1997. Structure and function of the plant alternative oxidase: its putative role in oxygen defence mechanisms. Bioscience Reports 17, 319–333.

Xie, Z., Chen, Z., 1999. Salicylic acid induces rapid inhibition of mitochondrial electron transport and oxidative phosphorylation in tobacco cells. Plant Physiol. 120, 217–225.