Comparison of defence responses to Botrytis cinerea infection in tomato plants propagated in vitro and grown in vivo

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(Received: 18.09.96)

Abstract

Defence reactions: $O_2^-$ generation, superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase activities after B. cinerea infection in tomato plants propagated in vitro and grown in vivo have been compared. Infection resulted in rapid $O_2^-$ generation. Superoxide dismutase activity increase was slower than $O_2^-$ response. In plants propagated in vitro catalase and guaiacol peroxidase activities after infection were induced less strongly than in plants grown in vivo. $K_2HPO_4$ pretreatment of plants grown in vitro enhanced significantly the activities of catalase and guaiacol peroxidase after infection. Slight restriction of B. cinerea infection development in in vitro propagated plants pretreated with $K_2HPO_4$ was observed.

INTRODUCTION

The use of in vitro culture methods in propagating many plant species of economic importance has increased considerably during the last decade. However, in vitro propagated plantlets in the phase of acclimatization to greenhouse conditions are highly susceptible to pathogen attack. One can assume that in the stage of acclimatization in vitro plantlets defence system is less strongly or not so rapidly mobilized than that of plants propagated by traditional methods. Therefore studies on the defence system of in vitro propagated plantlets have been undertaken and attempts have been made to induce their defence reactions.

It has been demonstrated that defence responses observed in infected plants can be also induced by elicitors (S c h n e i d e r a n d U l l r i c h , 1994). Elicitors include preparations of pathogenic microorganisms as well as abiotic compounds. Recently it has been found that some inorganic salt components of in vitro culture media, applied
at higher concentrations, may also act as elicitors (Irving and Kuć, 1990). Induction of plant defence reactions in response to elicitor treatment may lead to increased resistance to subsequent infections.

In this work we compared defence reactions of in vitro propagated tomato plantlets and plants grown from seeds in a growth chamber and tried to induce defence responses of in vitro multiplied plantlets using K$_2$HPO$_4$ as an elicitor. We examined O$_2^-$ generation and superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (PO) and ascorbate peroxidase (APX) activities as defence responses after B. cinerea infection.

**MATERIALS AND METHODS**

**Tomato plants.** Tomato plants (Lycopersicon esculentum Mill. cv. „Perkoz”) were grown from seeds in soil, in a growth chamber at 23°C and with 16 h photoperiod or were propagated in vitro on MS medium supplemented with 1 mg/l BAP and 0.05 mg/l IAA. Plantlets from in vitro culture were rooted for 1 week on MS medium with 1 mg/l NAA. Then plantlets were transferred to perlite and grown in a growth chamber at 23°C and with 16 h photoperiod. To induce defence responses one part of in vitro multiplied plantlets was rooted on the same medium supplemented with 5 mM K$_2$HPO$_4$.

**Botrytis cinerea infection.** Tomato plants grown from seeds were inoculated with fungal conidia at the age of 2 weeks and in vitro propagated plants, both K$_2$HPO$_4$ pretreated and non-treated, after transfer to perlite. B. cinerea conidial suspension contained 1 x 10$^6$ conidia in 1 ml. After inoculation plants were kept at 100% relative humidity. Leaves were examined for enzyme activity and O$_2^-$ content 1, 2, 3 and 5 days after inoculation. Control plants were examined simultaneously.

**Preparation of enzyme extracts.** The leaves were homogenized (1 : 5 w/v) in 1 M NaCl in 50 mM phosphate buffer pH 7.0 containing 1% PVP, 1 mM EDTA. For assay of APX extracts were prepared in the same medium containing 1 mM sodium ascorbate. The homogenate was centrifuged and the supernatant was used as enzyme extract to assay SOD, CAT, PO and APX activities.

**The detection of O$_2^-$** was based on its ability to reduce nitro blue tetrazolium (NBT) and was performed according to Doke et al. (1983). Five fresh leaf discs (Ø 0.8 cm) were immersed in 3 ml 0.01 M potassium phosphate buffer pH 7.8 containing 0.05% NBT and 10 mM NaN$_3$ for 1 hour. Then the mixture was heated at 85°C for 15 minutes and cooled rapidly. The discs’ activity to reduce NBT was expressed as increased absorbance per hour per disc.

**Enzyme assays.** The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Beauchamp and Fridovich as described by Hindawi et al. (1981). CAT activity was measured spectrophotometrically according to Hindawi et al. (1981) and that of guaiacol peroxidase as described by Maehly and Chance (1954). APX was determined
with sodium ascorbate as a substrate according to Nakano and Asada (1981) but we used 265 nm instead of 290 nm (an absorbance coefficient of 13.7 mM\(^{-1}\)cm\(^{-1}\)). All results are the means of three separate experiments.

RESULTS AND DISCUSSION

In tomato plants, both grown from seeds and propagated in vitro, B. cinerea infection resulted in rapid \(O_2^-\) formation (Fig. 1). However, the highest increase in \(O_2^-\) formation, about 200% of control level, was detected earlier in plants multiplied in vitro. The rate of SOD activity increase was slower than \(O_2^-\) response (Fig. 2). SOD activity reached the highest level only 3 days after B. cinerea inoculation when \(O_2^-\) production decreased significantly. The fall back of \(O_2^-\) content after its early increase could be at least partly the effect of increasing SOD activity. CAT activity increased gradually during infection development and reached the highest level 5 days after inoculation, both in plants grown in vivo and those multiplied in vitro (Fig. 3). However, during the whole examined period CAT induction was about 20% weaker in in vitro propagated plantlets in comparison with plants grown from seeds. B. cinerea infection resulted in a significant increase in PO activity in plants grown in vivo (Fig. 4). The activity increase was visible 1 day after inoculation and persisted for 5 days. The highest activity, about 290% of control, was detected 5 days after inoculation. In plants propagated in vitro PO activity increase was detected later and 5 days after inoculation was about 1.5 times lower than that in infected plants grown in vivo. On the other hand the induction of APX was observed earlier and was slightly stronger in plants propagated in vitro e.g. 3 days after inoculation the APX activity in plants cultured in vitro and grown in vivo was 160% and 120% of control, respectively (Fig. 5).

![Graph](image_url)

Fig. 1. Time course of \(O_2^-\) generation in B. cinerea infected tomato leaves
Fig. 2. Superoxide dismutase activity in tomato leaves after *B. cinerea* infection.

Fig. 3. Catalase activity in tomato leaves after *B. cinerea* infection.

Fig. 4. Guaiacol peroxidase activity in tomato leaves after *B. cinerea* infection.
It has been reported that active oxygen species and antioxidant enzymes may contribute to the multicomponent mechanism of disease resistance. O$_2^-$ generation may be directly involved in hypersensitive response (Doke, 1983). The antioxidant mechanisms that may contribute to plant pathogenesis include SOD, CAT and APX (Baker and Ordal, 1995). The cell wall bound peroxidases have been reported to oxidase NAD(P)H leading to the formation of O$_2^-$ and H$_2$O$_2$, the latter is used in the formation of lignin (Hammerschmidt, 1984). Our results seem to support the reports indicating that active oxygen species and antioxidant mechanisms: SOD, CAT and peroxidases may play a role in plant – pathogen interactions.

We observed that in in vitro propagated plantlets the induction of CAT and PO in response to infection was weaker than in plants grown from seeds. It seemed interesting to examine whether there is a correlation between the diminished activity of CAT and PO and the high susceptibility of in vitro propagated plantlets in the phase of acclimatization to in vivo conditions. We used K$_2$HPO$_4$ to elicit CAT and PO activity in in vitro cultured tomato plantlets. It has been reported that foliar sprays of oxalate and phosphate salts solutions protected cucumber plants against a broad range of fungi, bacteria and viruses (Mach et al., 1991) and phosphate and bicarbonate induced protection against powdery mildew on rose plants (Rueveni et al., 1994). However, the mechanism of protection induced by these chemicals remains unknown. Göttsche and Kuć (1989) have suggested that protection is triggered by a process involving the sequestering of calcium from host tissues.

We observed that in K$_2$HPO$_4$ treated plantlets the activity of CAT did not change significantly (Fig. 6) whereas PO activity was elicited on an average 30% above the control level (Fig. 7). In plantlets pretreated with K$_2$HPO$_4$ and then infected the activity of CAT, 1 day after inoculation, increased about 40% above the level in the infected, non-treated ones. The activity of PO after infection in plantlets pretreated with K$_2$HPO$_4$ was induced stronger than after infection without pretreatment 100% and 40%, 1 and 3 days after inoculation, respectively. We observed that infection development in K$_2$HPO$_4$ treated plantlets was slightly restricted as compared with non-pretreated
ones. It seems that enhanced CAT and PO activities are not sufficient to increase resistance of \textit{in vitro} propagated plantlets in the stage of acclimatization. It was reported that the lack of epicuticular wax on the leaves of \textit{in vitro} propagated plantlets is also associated with low survivability during hardening off (Torres, 1989).

![Graph showing Catalase activity](image1)

**Fig. 6.** Catalase activity in \textit{in vitro} propagated tomato leaves after K$_2$HPO$_4$ pretreatment and \textit{B. cinerea} infection.

![Graph showing Guaiacol Peroxidase activity](image2)

**Fig. 7.** Guaiacol peroxidase activity in \textit{in vitro} propagated tomato leaves after K$_2$HPO$_4$ pretreatment and \textit{B. cinerea} infection.

In conclusion our results indicated that the K$_2$HPO$_4$ pretreatment sensitizes plantlets from \textit{in vitro} culture resulting in more rapid increase in CAT and PO activities after infection. It seems possible that enhanced activities of CAT and PO after inoculation, induced in phosphate pretreated plantlets, may contribute to the slight restriction of \textit{B. cinerea} infection development observed.

\textbf{Acknowledgement:} This work has been supported by University of Łódź, Grant No 505/724.
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Porównanie reakcji obronnych po infekcji Botrytis cinerea u roślin pomidora otrzymywanych techniką in vitro i rozmnażanych z nasion.

Streszczenie

W pracy porównywano reakcje obronne: generowanie O2-, aktywność dysmutazy ponadlitenkowej (SOD), katalazy (CAT), peroksydazy guajakolowej (PO) i peroksydazy askorbinianowej (APX) w roślinach pomidora otrzymywanych techniką in vitro i rozmnażanych z nasion po inokulacji B. cinerea. Stwierdzono, że w wyniku infekcji następowala szybkie generowanie O2-. Wzrost aktywności SOD nastąpił później niż generowanie O2-. W odpowiedzi na infekcję rośliny rozmnażane in vitro reagowały słabszą indukcją CAT i PO. Przedinfekcyjne traktowanie roślin rozmnażanych in vitro K3HPO4 powodowało znacznie siśniejszą indukcję CAT i PO po infekcji. Obserwowano niewielkie ograniczenie rozwoju choroby w roślinach rozmnażanych in vitro pretraktowanych K2HPO4.