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FULL RESEARCH PAPER

# *In vitro* screening for sunflower (*Helianthus annuus*L.) resistant calli to *Diaporthe helianthi* fungal culture filtrate

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**Abstract** *Diaporthe helianthi* the causal agent of sunflower (*Helianthus annuus*) stem canker, causes significant reductions in yield and oil content in most sunflower-growing areas. With the aim of enhancing host resistance, we selected in vitro sunflower calli against culture filtrates of two pathogen isolates (7/96 and 101/96). This technique may be an effective and rapid tool to discriminate the most virulent *D. helianthi* isolate and to screen for host resistance in the early stage of a breeding programme. Further investigation on the mechanisms involved in defence pathways showed no induction of salicylic acid and pathogenesis-related proteins in calli, indicating that the host resistance is not associated with Systemic Acquired Resistance but probably other biochemical mechanisms.

**Keywords** Benzothiadiazole · Pathogenesis-related proteins · *Diaporthe helianthi* · Salicylic acid · Calli selection

**Abbreviations**

| | |
|---|---|
| CI | Callus induction medium |
| CM | Callus maintenance medium |
| 2,4 D | 2,4 Dichlorophenoxyacetic acid |
| MA | Malt agar |
| MS | Murashige & Skoog's medium |
| NAA | α-Naphthalenacetic acid |
| PR proteins | Pathogenesis-related proteins |
| SA | Salicylic acid |
| SAR | Systemic acquired resistance |
| V.I. | Variation index |

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## Introduction

*Diaporthe helianthi* (anamorph *Phomopsis helianthi*) (Muntanola-Cvetkovic et al. 1981) is the causal agent of sunflower stem canker, a major disease in most sunflower-growing areas. The fungus, which was isolated for the first time in 1979 in Yugoslavia (Acimovic and Straser 1981), has been subsequently reported in Australia and in many European, American and Asian countries (Vukojevic et al. 2001), where it causes significant losses in yield and oil content. By contrast, in Italy *D. helianthi* was noticed for the first time in 1987 (Zazzerini et al. 1988) and has since been found sporadically causing only minor production damage. The difference between the situation in Italy and other countries could be explained by the high variability existing among the different strains of the pathogen (Tosi et al. 2001). Several investigations showed that *D. helianthi* strains are distinguishable by morphological and

pathogenic features (Vukojevic et al. 1996; Viguie et al. 1999; Tosi et al. 2001) and by their ability to produce different amounts of phytotoxic compounds (Mazars et al. 1990; Avantaggiato et al. 1999).

Mazars et al. (1990, 1991) purified a phytotoxin called phomozin from *D. helianthi* culture filtrates and from infected sunflower seedlings; purified toxin, inoculated into sunflower leaves, produced alterations in the host tissues comparable to those observable during development of the disease, consisting of the formation of wide necrotic areas, encircled by a chlorotic halo. In other surveys, carried out with two Italian and two French strains of *D. helianthi* with different degrees of virulence, Avantaggiato et al. (1999) did not find phomozin but they purified two new phytotoxic compounds identified as *cis*-4,6-dihydroxymellein (*cis*-3-methyl-4,6,8-trihydroxy-3,4-dihydroisocumarin) and *trans*-4,6-dihydroxymellein (*trans*-3-methyl-4,6,8-trihydroxy-3,4-dihydroisocumarin). Phomozin or *cis*- and *trans*-4,6-dihydroxymelleins, however, show some structural similarities and they can all be considered as derivatives of orsellinic acid (Avantaggiato et al. 1999).

Since cultivars of *Helianthus annuus* resistant to stem canker do not currently exist, the aim of the present work was to investigate the possibility of screening sunflower calli for resistance to *D. helianthi* by recurrent selection on media amended with culture filtrates of the pathogen, taking advantage of somaclonal variation. As showed by Masirevec et al. (1988), *in vitro* selection may be, in fact, more sensitive than *in vivo* screening. Further surveys were carried out to characterize the factors involved in the resistance of sunflower calli to *D. helianthi* fungal filtrates: in particular, SA accumulation and PR proteins production were investigated.

## Materials and methods

### Fungal isolates

Two French isolates of *D. helianthi* (7/96 and 101/96) obtained from infected sunflower stems were kindly provided by J. Fayret (Laboratoire de Criptogamie, Université Paul Sabatier, Tolouse) and by the International Mycological Institute (IMI 318861), respectively. Both isolates were maintained on malt agar (MA) at 20°C. In previous *in vivo* pathogenicity tests and in bioassays for the production of *cis*- and *trans*-4,6 dihydroxymelleins, these isolates were the most (7/96) and the least (101/96) virulent, producing the highest (7/96) and the lowest (101/96) amounts of phytotoxins (Tosi et al. 2001; Tosi et al. 2002a).

### Production of fungal filtrates

Fungal filtrates of each *D. helianthi* isolate (7/96 and 101/96) were obtained by culturing 4–5 mycelial plugs (5 mm diam), taken from the edge of 10 day-old colonies grown on MA, in 500 ml flasks containing 250 ml of malt broth. Liquid cultures were incubated on a rotary shaker, in darkness, at 24 ± 2°C for 3 weeks. Each culture filtrate was collected by two successive filtrations through 0.45 μm and 0.20 μm Minisart microfilters. Sterile filtrates was stored at 4°C in aliquots of 100 ml. Before each use, fungal filtrates were tested for *cis*- and *trans*-4,6 dihydroxymellein contents as described by Avantaggiato et al. (1999).

### Sunflower callus induction

Sunflower (*H. annuus* cv. Ala) calli were induced and maintained as described by Tosi et al. (2002b). Briefly, achenes were surface-sterilized by immersion in 8% sodium hypochlorite for 2 min, rinsed in sterile deionized water and placed between a double layer of humid blotting-paper in Petri dishes incubated at 22 ± 2°C in darkness. After 2 days, germinated and dehulled achenes were sterilized in 1% sodium hypochlorite, aseptically transferred into glass tubes containing 15 ml of Murashige & Skoog's medium (MS) (Murashige and Skoog, 1962) and grown at 19 ± 2°C under 16 h daylength (40 $\mu Em^{-2} s^{-1}$). After 1 week, the cotyledons were excised, deprived of their basal portion and cut transversely into 2–3 pieces which were then transferred to MS medium supplemented with 5 $mgl^{-1}$ 2,4 D [CI (Callus Induction) medium] in Petri dishes; the pH of the medium was adjusted to 5.7. About two weeks later, callus nodules were transferred onto MS medium containing 0.1 $mgl^{-1}$ NAA [CM (Callus Maintenance) medium], pH 5.7. Calli were subcultured every month onto fresh CM medium.

*In vitro* selection for establishment of resistant calli and relative statistical analysis

Well-developed calli (approximately 0.5 g) were subjected to recurrent selection on CM medium containing sterile filtrate of either *D. helianthi* isolate. The pH of the fungal filtrates was adjusted to 5.7; they were added to CM medium cooled to 45°C. During each selection cycle, calli were incubated for 28 days at 19 ± 2°C under 16 h daylength (40 $\mu Em^{-2}$ $s^{-1}$). For each treatment, two Petri dishes, each divided into two compartments, were prepared. In each Petri dish two calli were incubated, for a total of four replications per treatment. In the first cycle, filtrate concentrations ranged from 5 to 40% (v/v), with 5% increments. Calli that survived the first selection cycle were transferred to the second cycle, where the fungal filtrates concentrations used ranged from 25 to 45% (v/v), with 5% increments, for isolate 7/96 and from 5 to 45% (v/v), with 5% increments, for isolate 101/96. In the second cycle, the lowest concentrations of the fungal filtrates used (25% v/v for isolate 7/96 culture filtrate and 5% v/v for isolate 101/96 culture filtrate) corresponded to the highest concentrations to which calli had survived at the end of the previous cycle. Calli surviving the second selection cycle were transferred to the third cycle, where the fungal filtrate concentrations used ranged from 35 to 60% (v/v), with 5% increments, for isolate 7/96 and from 20 to 50% (v/v), with 5% increments, for isolate 101/96. As described above, the lowest percentages of fungal filtrates used in the present cycle (35% v/v for isolate 7/96 culture filtrate and 20% v/v for isolate 101/96 culture filtrate) coincided with the highest percentages to which calli had survived at the end of the last cycle. Control calli were maintained in the same environmental conditions on CM medium with malt added in the concentrations reported for fungal filtrates and subcultured every 28 days on fresh medium.

Callus viability was assessed using two parameters: radial development (mm), as the average of two diameters of the callus measured at right angles to one another at the beginning and the end of every selection cycle, and the percentage of callus necrotic area, estimated weekly using an arbitrary colorimetric scale (Fig. 1). The choice of such parameters was dictated by the necessity to avoid destructive evaluation systems, as this was vital for recurrent selection.

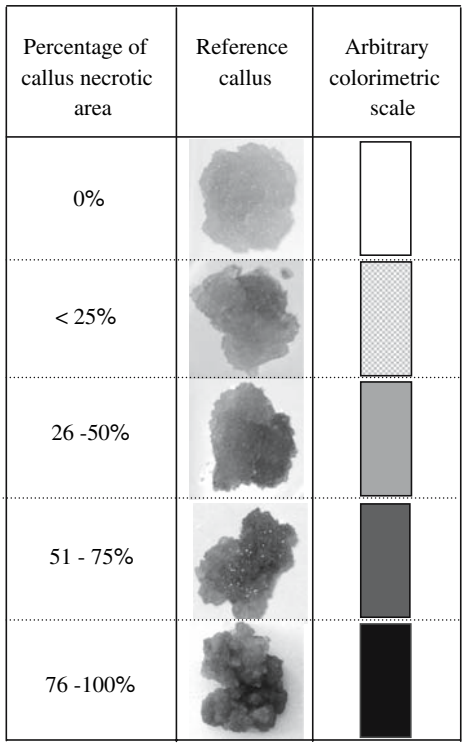


**Fig. 1** Arbitrary colorimetric scale for evaluation of callus viability

For every callus in selection the variation percentage of the radial development Variation Index (V.I.) was calculated as $[(r_f - r_i/r_i) \times 100]$, where $r_i$ and $r_f$ were, respectively, the radial development of the callus (in mm) at the beginning (i = initial) and at the end (f = final) of every selection cycle. This conversion was necessary because of differences between the diameter of the calli at the beginning of the selection cycles. The V.I. assumed positive or negative values respectively if callus diameter increased ($r_f > r_i$) or decreased ($r_f < r_i$) during the selection cycle. The obtained values (percentage of fungal filtrates added to substrate CM) were submitted to one-factor analysis of variance (ANOVA) for calli selected at different concentrations of every fungal filtrate. Four replications were used for each treatment. Multiple comparisons were carried out by Fisher's Protected LSD (Least Significant Difference). Statistical analyses were carried out separately for every cycle of selection.

### Treatments of sunflower calli in liquid culture with fungal filtrates and with benzothiadiazole

Well-developed sunflower calli (approximately 1 g), not previously subjected to recurrent selection, were incubated on a rotary shaker at 100 g, at 19 ± 2°C, under 16 h daylength (40 $\mu Em^{-2}$ $s^{-1}$), in 250 ml flasks containing 100 ml of CM liquid medium containing sterile filtrates of either *D. helianthi* isolate (7/96 and 101/96). Each fungal filtrate was added as one of two concentrations (10% and 50%) to CM liquid medium sterilized and cooled. Some calli were incubated in the same environmental conditions in 100 ml CM liquid medium supplemented with 0.25 mM benzothiadiazole (CGA 245704) ® Novartis, while control calli were incubated in CM liquid medium without fungal filtrates or benzothiadiazole. At 8, 24, 48 and 120 h after the beginning of the experiments, calli were collected and deep frozen (−80°C) before further analyses.

### Protein extraction and quantification

Proteins were extracted from sunflower calli proved to be resistant to *D. helianthi* culture filtrates after recurrent selection, from unselected sunflower calli treated in liquid culture with fungal filtrates and with benzothiadiazole and from control calli. Deep frozen calli (approximately 1 g) were ground in a mortar in 1 ml of extraction buffer (0.5 M sodium acetate, pH 5.2, containing 15 mM $\beta$-mercaptoethanol) (Jung et al. 1993), using quartz sand to aid extraction. After centrifugation of the homogenate at 10,000 g for 15 min at 4°C, the supernatant was collected and dialyzed overnight at 4°C in dialysis buffer (20 mM sodium acetate, pH 5.2, containing 15 mM $\beta$-mercaptoethanol) (Jung et al. 1993). Total protein quantification of sunflower calli extracts was performed as described by Bradford (1976), using the Biorad Bradford Kit (BIO-RAD) and BSA as standard.

### SDS-PAGE and western analyses

Electrophoresis on denaturing polyacrylamide gel was performed using the method of Laemmli (1970), with a 12.5% (w/v) resolving gel, pH 8.8 (Jung et al. 1993); 20 µg of protein were applied per lane. For immunological detection, proteins contained in the gel were electrotransferred for 2 h onto a nitrocellulose sheet (0.45 µm pore size) in western transfer solution containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 7.5, at a constant current of 400 mA. Antibody against tobacco PR 1b protein (Kauffmann, 1988) was used at a $10^{-3}$ (v/v) dilution while antibodies against tobacco PR 2 (Kauffmann et al. 1987), PR 3 (Lengrad et al. 1987) and PR 5 (Kauffmann et al. 1990) proteins were used at a $10^{-4}$ dilution. All antibodies were kindly supplied by S. Kauffmann (Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire des Plantes, Strasbourg, France). Protein extracts from salicylic acid-treated tobacco plants were used as the positive control of the reactions.

### Extraction and detection of free SA from sunflower calli and relative statistical analysis

SA was extracted from sunflower calli (1 g of fresh weight) treated in liquid culture with fungal filtrates of either *D. helianthi* isolates (7/96 and 101/96) or with 0.25 mM benzothiadiazole by homogenization in mortar with 2 ml 30% v/v methanol in water. The homogenate was centrifuged at 6,000 g for 2 min and extraction of the pellet repeated twice with 100% methanol. The recovery was evaluated by adding 160 µM *o*-anisinic acid as internal standard in the first extraction mixture. Subsequent to the three extractions, the supernatant was pooled and concentrated in vacuo, at room temperature, to 5 ml. After addition of 2 ml TCA 5%, the sample was filtered by 0.20 µm Minisart microfilters and analysed by HPLC (Jasco, Tokyo, Japan) using a Saulentechnik Hypersil ODS C18 column (12.5 × 4.00 mm). The sample was fractionated isocratically with methanol : acetic acid, 35:65 (v/v). SA was quantified by fluorescent detector (Ex 365 nm, Em 407 nm). The values of free SA (µg $g^{-1}$) were subjected to 2-way analysis of variance (ANOVA), (treatment × time). Four replications were used for each treatment. Multiple comparisons were made using Fisher's Protected LSD.

## Results

### *In vitro* selection for establishment of resistant calli to *Diaporthe helianthi* fungal filtrates

At the end of the first selection cycle, calli incubated on CM medium supplemented with *D. helianthi* isolate 7/96 culture filtrate at concentrations of 5 to 25% (v/v) remained white or showed necrosis extending to less than 25%; within this concentration range, only calli selected at 10% culture filtrate concentration showed a significant difference in the V.I. compared to control calli (Fig. 2a). Calli incubated on CM medium supplemented with the higher percentages (30, 35 and 40%) of the same fungal culture filtrate showed necrosis extending from 76 to 100% as soon as a week after the beginning of the selection; these calli showed negative values of V.I. due to the reduction of callus radial development during the selection cycle (Fig. 2a). Only calli incubated on CM medium supplemented with 5% (v/v) culture filtrate of *D. helianthi* isolate 101/96 survived the first selection cycle; calli selected at the higher concentrations of the filtrate showed necrosis ranging from 76 to 100%. The time taken for necrosis to develop was inversely related to culture filtrate concentration: after 21 days for selection on CM medium containing 10 or 15% culture filtrate, after 14 days for 20 or 25% culture filtrate and after almost 7 days on 30, 35 and 40% of the filtrate. The V.I. was comparable between control and survivor calli, while significant variations in radial development were observable between control and necrotic calli (Fig. 2b).

At the end of the second selection cycle, calli survived on CM medium with *D. helianthi* isolate 7/96 culture filtrate added at 25 to 40% (v/v); these calli remained white or showed cover necrosis of <25%. Calli selected on CM medium supplemented with 45% of the same fungal filtrate instead showed necrosis from 76 to 100%. At all culture filtrate concentrations there was no significant difference in V.I. from those for the control calli (Fig. 3a). On CM medium supplemented with *D. helianthi* isolate 101/96 culture filtrate at 5 to 15% and 30% (v/v) calli remained white or showed necrosis of <25%; moreover their radial development was comparable with control calli (Fig. 3b). Calli cultured on CM medium

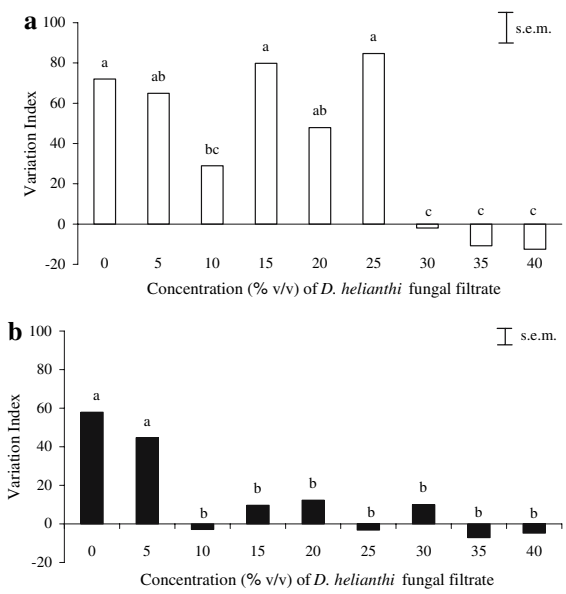


**Fig. 2** Variation Index (variation percentage of the radial development) in sunflower calli cv. Ala after the first cycle of selection on Callus Maintenance medium supplemented with different percentages of *D. helianthi* isolates 7/96 (a) and 101/96 (b) culture filtrates. (a) LSD ($P$ = 0.05) = 42.24 (b) LSD ($P$ = 0.05) = 24.37 s.e.m. = standard error of the mean

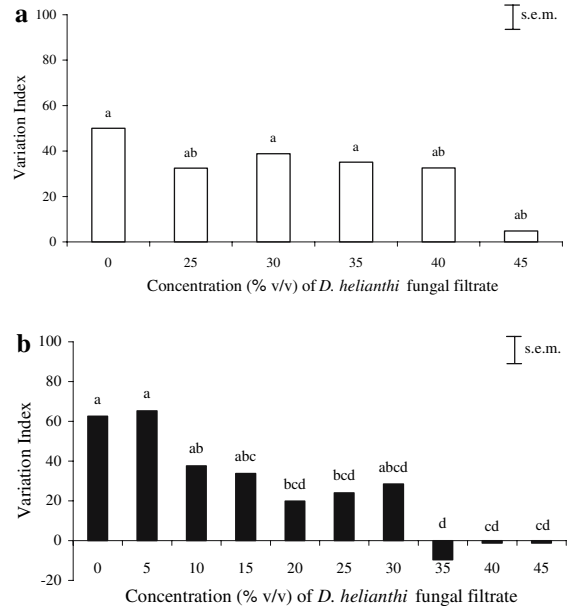


**Fig. 3** Variation Index (variation percentage of the radial development) in sunflower calli cv. Ala after the second cycle of selection on Callus Maintenance medium supplemented with different percentages of *D. helianthi* isolates 7/96 (a) and 101/96 (b) culture filtrates. (a) LSD ($P$ = 0.05) = 28.72. (b) LSD ($P$ = 0.05) = 38.18. s.e.m. = standard error of the mean

containing the same fungal filtrate at 20, 25, 35, 40 or 45% (v/v) however showed significantly lower radial development than the controls (Fig. 3b). Furthermore, at the end of the second selection cycle, calli incubated on CM medium supplemented with 25 or 30% (v/v) of *D. helianthi* isolate 101/96 culture filtrate showed necrosis ranging from 26 to 75% while calli selected on the higher concentrations (35, 40 and 45%) of the culture filtrate were completely necrotic and callus size had decreased (Fig. 3b).

At the end of the third cycle of selection, calli showed resistance to all concentrations (from 35 to 60% v/v) of *D. helianthi* isolate 7/96 culture filtrate to which they were exposed; calli incubated on 60% (v/v) culture filtrate showed necrosis ranging from 50 to 75% while all other concentrations produced calli which remained white or showed necrosis <50%. Only 50% and 55% (v/v) concentrations of fungal filtrate produced calli wich were not significantly different in size from control calli (Fig. 4a). At the end of the third selection cycle on CM medium supplemented with different concentrations of *D. helianthi* isolate 101/96 culture filtrate, calli survived at the lower percentages (from 20 to 35%) of the filtrate while they were completely necrotic at the higher percentages (from 40 to 50%). Only calli incubated on medium added with 35 and 50% of fungal filtrate showed growth significantly different from that of control calli (Fig. 4b).

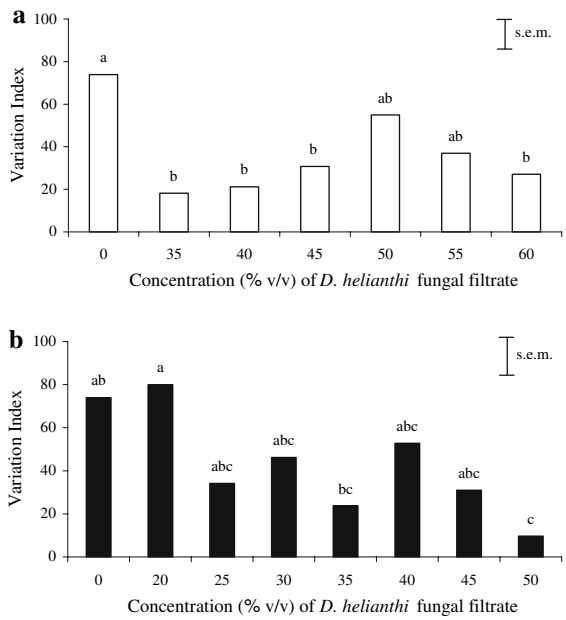


**Fig. 4** Variation Index (variation percentage of the radial development) in sunflower calli cv. Ala after the third cycle of selection on Callus Maintenance medium supplemented with different percentages of *D. helianthi* isolates 7/96 (a) and 101/96 (b) culture filtrates. (a) LSD ($P$ = 0.05) = 39.89 (b) LSD ($P$ = 0.05) = 51.07 s.e.m. = standard error of the mean

### SDS-PAGE and western analyses on sunflower calli cv. Ala

PR proteins were not detected on sunflower calli cv. Ala incubated in CM liquid medium supplemented with 10 or 50% (v/v) of *D. helianthi* isolate 7/96 culture filtrates, after 8, 24, 48 h and 5 days from the beginning of treatment (Fig. 5). The same result was obtained from calli incubated in CM liquid medium added with 0.25 mM benzothiadiazole or with 10 or 50% (v/v) of isolate 101/96 culture filtrates, after 8, 24, 48 h and 5 days from the beginning of treatment. Moreover, PR proteins were not detected in sunflower calli that showed resistance to *D. helianthi* fungal filtrates (isolate 7/96 and 101/96) after every cycle of recurrent selection. PR proteins were instead detected in SA- treated tobacco samples, used as the positive controls of the study.

### Free SA level in sunflower calli cv. Ala treated in liquid medium with *D. helianthi* fungal filtrates and with benzothiadiazole

Sunflower calli treated in liquid culture with 0.25 mM benzothiadiazole or with 10 or 50% (v/v) concentrations of *D. helianthi* fungal filtrates (isolate 7/96 or 101/96) did not show significant variation in free SA concentration compared to the control calli ($F$ value = 1.10; $P$ = 0.39) at any time (8, 24, 48 h and 5 days) from the beginning of treatment. Moreover, no significant variations in the level of free SA were detectable for each treatment at different times.

## Discussion

Our investigations showed that sunflower calli reacted positively to recurrent selection since the degree of resistance (expressed as concentration of each fungal filtrate to which they were resistant) increased as selection proceeded. The variation of the

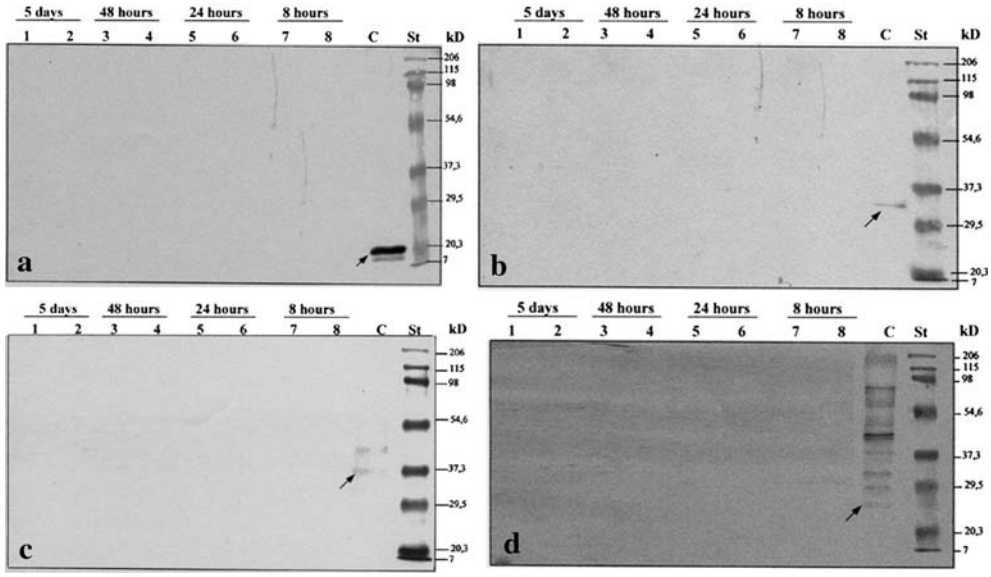


**Fig. 5** Western analyses on PR 1 (a), PR 2 (b), PR 3 (c) and PR 5 (d) proteins extracted from sunflower calli cv. Ala at different times from the beginning of treatment in liquid culture with 50% (v/v) of *Diaporthe helianthi* isolate 7/96 culture filtrate (samples 1, 3, 5, 7) and with 10% (v/v) of the same fungal filtrates (samples 2, 4, 6, 8). In the salicylic acid-treated tobacco plants, used as positive control (C), the proteins were just detectable (arrow). A Broad range standard (St) was used as molecular weight marker. 20 μg of protein were applied per lane

callus size was not always proportional to the fungal filtrate concentration in the selection medium and this phenomenon could be explained only by the variability existing between the cells within the calli. The reduction of callus size in necrotic calli was presumably due to degeneration and dehydration of cells incubated on media with higher concentrations of culture filtrate.

While Avantaggiato et al. (1999) showed a positive correlation between the content of *cis*- and *trans*-4,6- dihydroxymelleins in *D. helianthi* culture filtrates and the level of alterations that were produced when inoculated *in vivo* into the sunflower tissues, the present investigation showed no such correlation *in vitro*; calli of sunflower cv. Ala showed a good degree of resistance towards culture filtrate of isolate 7/96, which contained a high concentration of both toxins, while they were more susceptible to culture filtrate of isolate 101/96, which was relatively poor in dihydroxymelleins. Thorough examinations would be necessary in an attempt to determine the composition of both fungal filtrates (7/96 and 101/96) and to identify differential components from *cis*- and *trans*-4,6- dihydroxymelleins, putatively responsible for the greater toxicity of the *D. helianthi* isolate 101/96 sterile filtrate. However the recurrent selection against fungal filtrates enabled resistant calli to be obtained, starting from a susceptible sunflower cultivar (Ala), from which it may be possible to regenerate sunflower plants resistant to stem canker (Greco et al. 1984). This technique may provide an additional source of resistance in sunflower selection programmes; moreover *in vitro* selection seems to be an effective and quick tool to discriminate aggressiveness (virulence) among *D. helianthi* isolates.

The sunflower calli exhibiting toxin resistance showed neither SA accumulation nor PR protein production. This was similar to the finding of Prachi et al. (2002), who did not find PR 1 protein in calli of ginger (*Zingiber officinale*) treated with SA, and Katz et al. (1998), who did not observe SA accumulation in parsley (*Petroselinum crispum*) cell cultures treated with benzothiadiazole. Since SA accumulation and PR protein induction are commonly used as markers of SAR, the results here suggest that the resistance of sunflower calli cv. Ala to *D. helianthi* culture filtrate was not SAR. Probably other factors, different from PR proteins, are involved in this resistance as shown in studies carried out on calli of *Citrus limon* and *Vitis vinifera* Chardonnay selected

against pathogen culture filtrates, where the resistance was related to enhanced production of chitinase and glucanase (Gentile et al. 1993; Jayasankar et al. 2000).

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