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Indole-3-acetic acid attenuates the fungal lesions in infected potato tubers

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Received 20 October 2005; revised 25 November 2005

doi: 10.1111/j.1399-3054.2006.00667.x

In this report, we demonstrated that potato tubers pre-treated with 100 μ*M* of indole-3-acetic acid (IAA) and then inoculated with *Fusarium solani* f. sp. *eumartii* (*F. eumartii*) showed a decrease in the fungal lesion compared with non-IAA pre-treated and inoculated tubers. Consequently, we tested the addition of different concentrations of IAA on the fungal growth and on the proteolytic activity when the fungus was grown in liquid culture medium. Fungal growth did not change under different phytohormone concentrations, but the activity of *Fusarium* extracellular serine protease (FESP) clearly decreased. The inhibition of FESP activity by IAA was dose dependent. Moreover, FESP as well as others extracellular pectinolytic activities detected in IAA pre-treated and inoculated tubers decreased compared with controls. In addition, the ability of IAA production by *F. eumartii* was tested, and the role of IAA on potato–*F. eumartii* interaction is discussed.

Introduction

Fusarium solani (Mart.) Sacc. f. sp. *eumartii* (Carp.) Snyder et Hanser (*F. eumartii*) is a phytopathogenic fungus that causes dry rot, one of the most economically important post-harvest disease of potato tubers. The infected tubers show lesions on their surfaces that extend deeply in the tissue producing dry rot. Tissue rotting shows internal caves covered by mycelium (Godoy 2000). It has been reported that phytopathogenic fungi, such as *F. eumartii*, secrete a broad range of hydrolytic enzymes such as cellulases, pectinases, xylanases and proteases that are involved in penetration and colonization of host plant tissues during infection (Walton 1994). Previously, we had purified and characterized an extracellular serine protease produced by *F. eumartii*, called *Fusarium* extracellular serine protease (FESP) (Olivieri 2002). In addition, we proved that FESP is related with the fungal colonization of the potato tissue suggesting that a combined action of this and other hydrolytic enzymes could be, at least in part, responsible for the fungal lesions in the potato tubers (Olivieri et al. 2004).

Indole-3-acetic acid (IAA) is a phytohormone synthesized also by bacteria and fungi. In plants, it is the most abundant and widespread auxin. IAA is implicated in a wide range of developmental and growth responses, and it is also thought to be involved in host–pathogen interactions (Hamill 1993). Several microbial pathogens use phytohormones to alter physiological conditions in the host plant, such as *Agrobacterium tumefaciens* that causes plant neoplastic disease (Hooykaas and Schilperoort 1992). In addition, it has been reported that wounding elicits an important increase in the auxin levels of the plant tissues (Yamada 1993).

Abbreviations – FESP, *Fusarium* *e*xtracellular *s*erine *p*rotease; GC-EIMS-SIM, gas chromatography-electron *i*mpact *m*ass *s*pectrometry-*s*elected *i*on *m*onitoring; IAA, indole-3-acetic acid; IWF, intercellular washing fluid; PCIB, α-(*p*-chlorophenoxy) isobutyric acid; PDA, potato dextrose agar; PM, potato medium; RM, Richard medium; SD, standard deviation; W, wounding.

Martinez-Noël et al. (2001) showed that IAA attenuates the lesions of potato leaves infected with *Phytophthora infestants*. Prusty et al. (2004) suggested that the presence of IAA at the plant-wounded site makes it attractive for fungi. In addition, the authors described that high IAA concentrations inhibit the growth of *Saccharomyces cerevisiae*, whereas at lower concentrations (2 μ*M*), it induces adhesion and invasive growth.

The aim of this work was to evaluate the action of exogenous IAA on the potato–*F. eumartii* interaction. Noticeably, when potato tubers were pre-treated with 100 μ*M* IAA and then inoculated with *F. eumartii*, the fungal lesions decreased compared with non-IAA pre-treated and inoculated tubers. This fact could be explained, at least in part, by the fact that determined IAA concentrations do not affect fungal growth, but they inhibit FESP and others hydrolytic activities. Consequently, it is proposed that the IAA modulates extracellular hydrolytic enzymes, and thereafter, it may regulate *F. eumartii* pathogenicity in a dose-dependent manner, attenuating the fungal lesions in the host tissue.

Materials and methods

Plant and fungal materials

The experiments were performed using potato tubers of *Solanum tuberosum* L., cv. Pampeana, which is susceptible to the fungus. *F. solani* (Mart.) Sacc. f. sp. *eumartii* (Carp.) Snyder et Hanser (*F. eumartii*), isolate 3122, from EEA-Balcarce INTA Collection (Argentina) was grown during 3 weeks at 25°C in Petri dishes on solid potato dextrose agar (PDA) medium.

Fungal growth in liquid media

To assess IAA production by *F. eumartii*, the fungus was grown in two different liquid culture media: Richard medium (RM) and potato medium (PM). RM is a chemically defined medium that contains 1% (w/v) KNO_3, 0.5% (w/v) KH_2PO_4, 0.25% (w/v) $MgSO_4 \cdot 7H_2O$, 0.002% (w/v) $FeCl_3$ and 5% (w/v) sucrose. PM is prepared by boiling 200 mg l^{-1} of potato tubers during 40 min in distilled water, and after filtration through filter sheets, the liquid was autoclaved and then used. Volumes of 125 ml of each liquid culture media were inoculated with a 0.4-cm diameter disk of fungus grown in PDA. Fungal cultures were incubated for 7 and 14 days at 25°C in an orbital shaker at 180 r.p.m. in darkness. Filtrates were freeze dried and stored at 20°C. Mycelia were dried at 65°C for 3 h, and the mycelium dry weight was estimated.

For the measurement of proteolytic activity, the fungus was grown in RM supplemented with 0.1 mg ml^{-1} of denatured potato proteins instead of sucrose for 4–7 days at 25°C in an orbital shaker at 180 r.p.m. in darkness. As described above, the mycelium dry weight was calculated as estimation of the fungal growth.

Tuber inoculation procedures and hormone treatments

Tubers were acclimatized during 24 h at 25°C in darkness. After that, they were washed with water, surface sterilized by immersion in 0.5% (v/v) sodium hypochlorite for 5 min and then rinsed with sterile water. Tubers were inoculated with a 0.4-cm diameter disk of fungus grown in PDA and introduced in the potato cortical tissue by the hollow punch method described by Radtke and Escande (1973). As this method causes mechanical injury to the tuber tissue (wounding), control tubers were wounded and inoculated with sterile PDA. After inoculation, tubers were stored for 7 or 13 days at 25°C, then cut and analysed.

For hormone pre-treatments, 40 μl of 100 μ*M* IAA or 40 μl of 100 μ*M* IAA and 200 μ*M* α-(*p*-chlorophenoxy) isobutyric acid (PCIB) were applied on wounded tubers. After 3 h, fungal inoculations were performed as described above. Controls were pre-treated with a solution containing 0.2% (v/v) ethanol.

Assessment of lesion area in potato tubers

Tubers were transversally cut, approximately in the middle of the lesion site. Areas of the cross-section showing visible dry rot were recorded. Surface of rot areas were quantified by ImageJ 1.33u (Wayne Rasband, National Institutes of Health) (Olivieri et al. 2004).

Extraction and measurement of IAA

For IAA measurements, 1 g of potato tuber fresh weight collected from the area surrounding the inoculation site or 125 ml of fungal culture filtrates were extracted with 100 μl of buffered (pH 7.0) 65% (v/v) isopropanol and 10 ng ^{13}C6-IAA (JD Cohen, University of Minnesota-Saint Paul) on ice (allowing 1 h equilibration for internal standards). Sample was diluted 10-fold with distilled water and loaded without vacuum on NH_2 column [previously conditioned with hexane, acetonitrile, distilled water and then with imidazole buffer (pH 7.0) and washed with distilled water]. After 5 min, the solvent (in which the sample was diluted) was pulled out with vacuum (30 seg), then the column was sequentially rinsed with hexane, ethyl acetate, acetonitrile and

methanol, and finally the sample was eluted with three fractions of 600 μl 0.25% (v/v) phosphoric acid. The elute from the NH_2 column was then loaded on to C18 minicolumns [previously washed with distilled water, 80% (v/v) methanol, 1% (v/v) acetic acid and 0.25% (v/v) phosphoric acid]. The column was rinsed with distilled water pH 3.0, and the sample finally eluted with 300 μl methanol. Samples were dried out under N_2 stream and derivatized with fresh ethereal diazomethane prior to GC/MS analysis. Quantitative IAA analysis was carried on a capillary gas chromatography-electron impact mass spectrometry-selected ion monitoring (GC-EIMS-SIM) system Agilent HP 6890 GC interfaced to a HP 5972 Mass Selective Detector, equipped with a 30-m HP-5 capillary column (film thickness 0.2 μm). Carrier gas was He at 1 ml min^{-1}, GC injector was at 250°C, and oven temperature was increased from 90 to 260°C at a rate of 4°C min^{-1}. Ions monitored were m/z 130 and 136 for the base peak (quinolinium ion) and 189 and 195 for the molecular ion of the methyl-IAA and methyl [$^{13}C_6$]-IAA, respectively. The ratios 130:136 and 189:195 were used to calculate endogenous levels of IAA and to verify the analysis by triplicate.

Isolation of intercellular washing fluids

To measure FESP and pectinolyitc activities, intercellular washing fluids (IWF) from potato tubers were extracted as previously described (Olivieri et al. 1998).

Measurements of FESP activity

FESP activity was measured in 50 m*M* Tris-HCl, pH 8.0, using 5 mg of azocasein as substrate in a final volume of 0.5 ml. After incubation at 42°C for different times, reactions were stopped by addition of 0.5 ml of 10% (v/v) trichloroacetic acid (TCA). After 30 min at 4°C, undigested material was removed by centrifugation at 3000 *g* for 15 min. Proteolytic activity was estimated as the increase of absorbance at 335 nm of the TCA-soluble fraction. One unit of activity represents the amount of enzyme that produces a change of 1.0 in absorbance at 335 nm during 1 h at 42°C.

Analysis of pectinolytic activity by 10% SDS-PAGE

Pectinolytic activity was measured in gels according to Ried and Collmer (1985) with the following modifications. SDS-PAGE was performed on slab gels using 10% (w/v) acrylamide and 0.5% (w/v) pectin co-polymerized. Samples were treated with sample buffer (Laemmli 1970) without boiling before PAGE. After running, the gel was incubated during 2 h at 30°C in 50 m*M* Tris-HCl pH 7.5 and 0.1 m*M* $CaCl_2$. Activity bands were visualized by staining the gels for 1 h in 0.03% (w/v) Ruthenium red. At working pH, the colourless bands represent mainly polygalacturonase activity; however, pectatolyase activities should not be discarded (Ried and Collmer 1985).

Results

IAA application attenuates the extent of fungal damage and affects FESP activity in infected potato tubers

To examine the effect of exogenous IAA application on potato tubers upon inoculation with *F. eumartii*, tubers were submitted to the following treatments: wounding (W), wounding and inoculation with *F. eumartii* (W + *F. eumartii*), wounding and 100 μ*M* IAA application (W + IAA), wounding, 100 μ*M* IAA application and then inoculation with *F. eumartii* (W + IAA + *F. eumartii*). After 13 days upon treatment, the typical symptoms of dry rot caused by *F. eumartii* was observed in W + *F. eumartii* and in W + IAA + *F. eumartii* tubers (Fig. 1, lower panels). However, the extent of damage caused by inoculation with *F. eumartii* was larger in W + *F. eumartii* tubers than in W + IAA + *F. eumartii* ones (Fig. 1, lower panels, left and right, respectively). These differences were estimated by measuring the lesion area as described in *Materials and methods*. The results indicate that the lesions on W + IAA + *F. eumartii* tubers were reduced 41% compared with W + *F. eumartii* tubers. Similar results were obtained when tubers were pre-treated with 50 μ*M* IAA (data not shown). Control tubers (W or W + IAA) did not show fungal lesions (Fig. 1, upper panels). To know if the exogenous IAA may act as a fungistatic compound affecting fungal growth, we quantified *F. eumartii* biomass. Thus, the fungus was grown in liquid culture media containing different IAA concentrations (1.10^{-3} to 500 μ*M*). Clearly, the fungal biomass did not change among the broad range of IAA concentrations used in this work (Fig. 2, striped bars), suggesting that fungal growth is not affected by exogenous IAA. Previously, Olivieri et al. (1998) reported that *F. eumartii* is able to produce FESP in liquid culture as well as in IWF of infected tubers (Olivieri et al. 1998). These evidences led us to ask about the action of exogenous IAA on FESP activity. This activity was measured in *F. eumartii* culture media. FESP activity was expressed as values relative to control (medium without IAA). Fig. 2 (white bars) shows that FESP activity almost did not change with the addition of 1.10^{-3} to 1 μ*M* of IAA-containing medium.

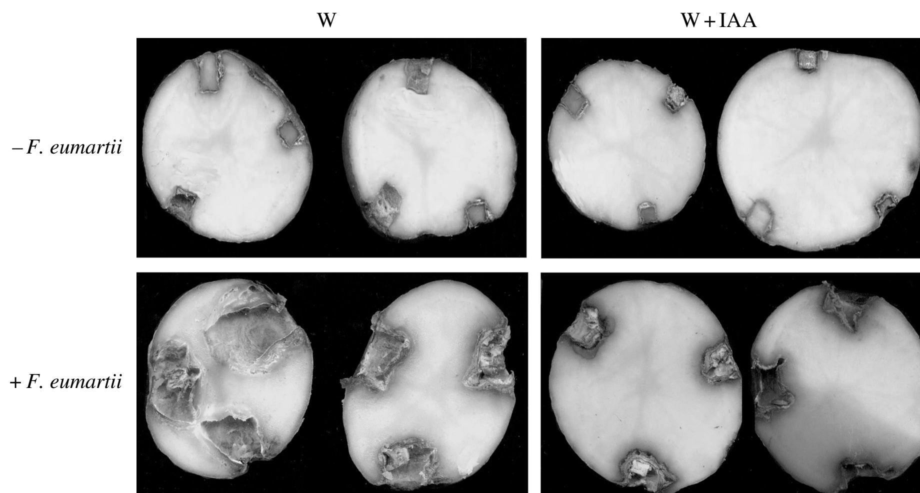

Fig. 1. Visual symptoms of potato tubers pre-treated with indole-3-acetic acid (IAA) and inoculated with *Fusarium eumartii.* Wounded tubers were pre-treated with control solution (W) or 100 μ*M* IAA (W + IAA) and then inoculated with *F. eumartii:* (W + *F. eumartii*) and (W + IAA + *F. eumartii*). Pictures of the transversally cut tubers were taken 13 days after inoculation.

However, with 10, 100 or 500 μ*M* IAA, FESP activity drastically decreased indicating that IAA modulates this activity in a dose-dependent manner.

FESP activity was also measured in IWF of treated potato tubers. Fig. 3A shows that in IWF of W or W + IAA tubers (controls), FESP activity was not detected. When potato tubers were infected with *F. eumartii* (W + *F. eumartii*) FESP activity increased. In the case of IAA treatment plus inoculation with *F. eumartii* (W + IAA + *F. eumartii*), FESP activity decreased almost 60% compared with W + *F. eumartii.* We also assayed the action of PCIB (a competitive inhibitor of IAA with no auxin-like activity) on FESP activity. As expected, in inoculated tubers pre-treated with IAA plus PCIB (W + IAA + PCIB + *F. eumartii*), the IAA effect on FESP activity was abolished. To test if FESP activity is inhibited in vitro by IAA, we added 100 μ*M* IAA in the test tube containing IWF extracts from infected tubers. FESP activity did not change by the addition of IAA, suggesting that the hormone is not a direct protease inhibitor and that the inhibition may be because of a possible hormonal regulation of the proteolytic activity (data not shown).

We also assayed the action of exogenous IAA on other fungal hydrolytic enzymes. The pectinolytic activities visualized by a zymogram showed a similar pattern of FESP activity. Fig. 3B shows that the extracellular pectinolytic activities accumulated in potato tubers upon infection by *F. eumartii* are reduced by IAA pre-treatment (W + IAA + *F. eumartii*) compared with control pre-treatment (W + *F. eumartii*). We noted that the experimental conditions used in this zymogram did not

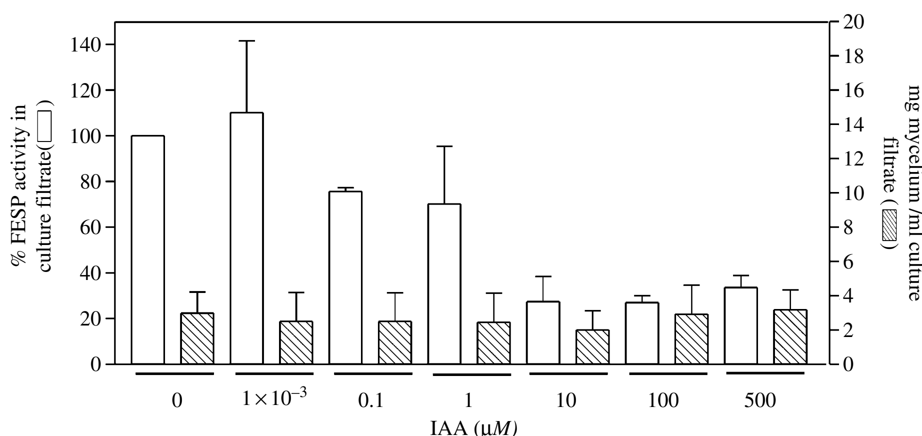

Fig. 2. Effect of exogenous indole-3-acetic acid (IAA) on fungal growth and *Fusarium* extracellular serine protease (FESP) activity in liquid culture media. Fungus was grown in RM containing denatured potato proteins and supplemented with different concentrations of IAA for 4–6 days at 25°C in darkness. FESP activity of the culture filtrate was measured using azocasein as substrate. 100% of proteolytic activity corresponds to the activity detected in the medium without IAA. The units of activity are expressed for mg^{-1} mycelium. The fungal dry weight was estimated after the mycelium was dried at 65°C for 3 h. Values are the mean of at least three independent experiments. Error bars represent the SD.

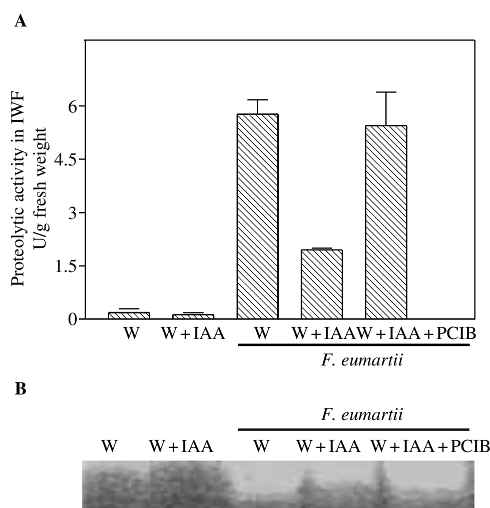

Fig. 3. Effect of indole-3-acetic acid (IAA) pre-treatment on *Fusarium* extracellular serine protease (FESP) activity accumulated in intercellular washing fluid (IWF) of potato tubers. (A) FESP activity was measured in IWF from tubers submitted to the following treatment: wounded (W); wounded plus 100 μ*M* IAA pre-treated and then inoculated (W + IAA + *Fusarium eumartii*) or not with *F. eumartii* (W + IAA); wounded plus control solution pre-treated and then inoculated (W + *F. eumartii*) and wounded plus 100 μ*M* IAA plus 200 μ*M* α-(*p*-chlorophenoxy) isobutyric acid (PCIB) pre-treated and then inoculated with *F. eumartii* tubers (W + IAA + PCIB + *F. eumartii*). FESP activity was measured using azocasein as substrate. One unit of activity represents the amount of enzyme that produces a change of 1.0 in absorbance at 335 nm during 1 h at 42°C. (B) Zymogram of pectinolytic activities in IWF. Proteins were loaded on 10% SDS-PAGE containing 0.5% (w/v) pectin co-polymerized followed by Ruthenium red staining. Values are the mean of at least three independent experiments. Error bars represent the SD.

allow us to distinguish between polygalacturonase and pectatelyase activities. Finally, we concluded that exogenous IAA affects different extracellular hydrolytic activities.

Endogenous free IAA levels change at the tissue surrounding the inoculation sites of potato tubers and in *F. eumartii* liquid cultures

As shown in Fig. 1, the exogenous application of IAA affects the potato–*F. eumartii* interaction. At this point, we considered to analyse the endogenous level of free IAA present at the tissue surrounding the inoculated site. The free IAA level in healthy (non-wounded and non-inoculated; H), wounded (W) and wounded plus inoculated tubers (W + *F. eumartii*) was measured. Fig. 4 shows that the endogenous IAA content increased at least three-fold after infection compared with healthy tubers. However, the IAA level almost did not change by wounding treatment. These results indicate that, in potato tubers upon fungal stress, the free IAA content clearly increased. This fact led us to think that the IAA detected in infected tubers may be because of the presence of *F. eumartii* on the host tissue.

To get a clue about the ability of *F. eumartii* to produce IAA, we grew the fungus in both liquid PM and chemically defined RM. IAA was characterized by full scan EIMS and measured in the fungal filtrates of both media at 7 and 14 days of growth by SIM isotopic dilution. The results clearly indicated that *F. eumartii* produces IAA in PM medium. In Fig. 5, the values expressed as microgram IAA per gram dry weight are equivalent to 61.7 ± 7.4 and 60.4 ± 2.3 ng ml^{-1} at 7 and 14 days, respectively. The IAA level detected in this medium without fungus was approximately 181 ± 8.5 pg ml^{-1}. IAA levels detected in MR medium were 105.2 ± 48.1 pg ml^{-1} in absence of fungus and 285.8 ± 34.8 and 314.2 ± 2.5 pg ml^{-1} at 7 and 14 days, respectively.

Discussion

In this work, we reported that potato tubers pre-treated with 100 μ*M* IAA and then inoculated with *F. eumartii* (W + IAA + *F. eumartii*) showed less damage because of fungal infection compared with non-IAA pre-treated tubers (W + *F. eumartii*) (Fig. 1). Although auxins have multiple effects on plant cells and it is difficult to establish relationships between IAA and pathogenesis, there are many reports describing that IAA is necessary for pathogenicity (Smidt and Kosuge 1978, Furukawa and Syono 2003). Cohen et al. (2002) reported that

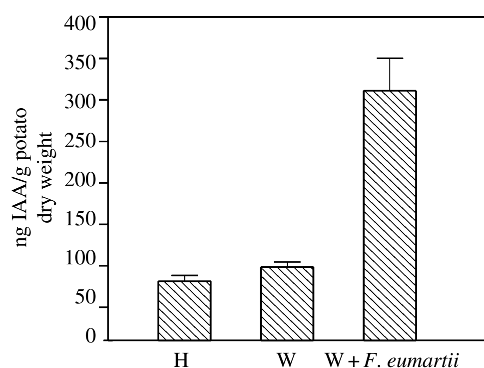

Fig. 4. Indole-3-acetic acid (IAA) levels of healthy (H), wounded (W) or wounded plus *Fusarium eumartii* inoculated (W + *F. eumartii*) potato tubers. Tubers were wounded or wounded and inoculated for 24 h with *F. eumartii*. Tissue surrounding the inoculation site was processed and IAA levels were analysed by gas chromatography-electron impact mass spectrometry-selected ion monitoring. Values are the mean of three replicates. Error bars represent the SD.

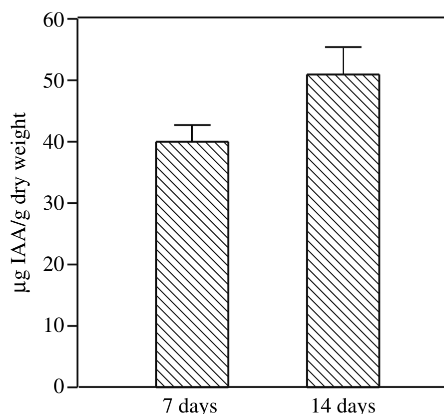

Fig. 5. Indole-3-acetic acid (IAA) levels produced by *Fusarium eumartii* in liquid culture media. Fungus was grown in potato media for 7 and 14 days at 25°C in darkness. After that, culture filtrates were freeze dried, and IAA levels were measured by gas chromatography-electron impact mass spectrometry-selected ion monitoring. Values are the mean of three replicates. Error bars represent the SD.

transgenic *Fusarium oxysporum* and *Fusarium arthrosporioides* overproducing IAA confers hypervirulence to plant pathogens. In contrast, Martinez-Noël et al. (2001) suggested that IAA may have antifungal properties in potato plants. In our system, exogenous application of IAA at the wounded and inoculated site restricts the fungal damage. Consequently, we decided to analyse some biochemical components which may correlate with the fungal infection on potato tissue. Among the molecules responsible for the *F. eumartii* pathogenicity, FESP has been previously postulated (Olivieri et al. 2004). Thus, FESP activity was measured in fungal culture filtrates containing different concentrations of exogenous IAA. Relatively low IAA concentrations (1.10^{-3} to 1 μM) had not effected on FESP activity, but when the concentration increased to a range between 10 and 500 μM, FESP activity drastically decreased. In contrast, the application of a wide range of IAA concentrations (1.10^{-3} to 500 μM) in liquid cultures did not affect fungal growth (Fig. 2). Therefore, we conclude that the IAA dose-dependent action on FESP activity suggests a hormonal regulation. In addition, we compared FESP activity accumulated in planta. The activity detected in IWFs of infected tissue decreased in 100 μM IAA pre-treated tubers compared with non-IAA pre-treated ones (Fig. 3A). In conclusion, the results indicated that the attenuate response of tubers against fungal attack occurs in parallel with the decrease of FESP activity. Like FESP, pectinolytic activity significantly decreased in tubers pre-treated with 100 μM IAA and then infected (Fig. 3B). This fact indicates that IAA alters different fungal hydrolytic enzymes rather than it acts as an inhibitor of the fungal growth. Roco and Pérez (2001) described the partial inhibition of an endopolygalacturonase of *Alternaria alternata* by IAA, suggesting that the hormone may interfere with the secretion of the enzyme or its level. Probably, *F. eumartii* extracellular enzymatic activities are also regulated in a similar way.

On the other hand, in an attempt to exploit the effect of exogenous IAA on the potato–*F. eumartii* interaction, we decided to analyse the endogenous IAA level in infected potato tubers. An increase of three-fold was detected in infected tubers compared with controls, suggesting that *F. eumartii* may produce IAA during its colonization on plant tissue. However, the plant contribution to this increase may not be discarded. Therefore, we asked if the fungus is able to produce IAA. Free IAA level in both, an unspecific and a chemically defined liquid media (PM and RM, respectively) was quantified. The IAA concentration in both media without the fungus was insignificant. IAA produced by *F. eumartii* was clear in PM, whereas no significant IAA levels were detected in RM, suggesting a clear dependence of IAA production on the chemical composition of each medium (Fig. 5). Several in vitro studies with other fungi demonstrated that some micro-organisms can produce variable amounts of IAA according to their growth in the presence or not of physiological precursors (Rey et al. 2001). Maor et al. (2004) described that the IAA biosynthesis by *Colletotrichum gloeosporioides* f. sp. *aeschynomene* is strictly dependent on external tryptophan sources and that infected plants contain elevated levels of IAA compared with the levels of uninfected plants. According to these results, the PM filtrate might be a more abundant source of IAA precursors.

At this point, we do not have enough evidences to correlate our findings on the action of exogenous IAA and the role of this hormone in planta during the plant–pahtogen interaction. We can conclude that high concentrations of IAA (10 or 100 μM) decrease the activity of different extracellular hydrolytic enzymes which has been associated with the fungal lesion or dry rot on potato tubers, and the manipulation of the IAA levels might have an impact on the agricultural fields. Briefly, in our experimental system, *F. eumartii* infects potato tissue (Fig. 1) and apparently it produces IAA during the process (Figs 4 and 5). However, when the fungus grew in the presence of high IAA concentrations, its pathogenicity decreases (Fig. 1, lower right panel), making evident that the IAA effect differs in each conditions. Consequently, we speculate that the endogenous IAA level may be different from those exogenously applied. Other possible explanation could be related with the timing of IAA

production by *F. eumartii* (24 h post-inoculation) and the moment of exogenous IAA application (3 h before inoculation). However, we still require a broader understanding on how the homeostasis of IAA regulates this plant–pathogen interaction, and further studies are needed to elucidate its role.

Acknowledgements – We thank Dr AV Godoy for the critical reading of the manuscript. This work was supported by grants from the IFS (Sweden), UNMDP, CONICET, CIC, ANPCyT (Argentina). CAC and RB are members of CONICET. MCT and FPO were recipients of fellowships from the same institution.

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Edited by C. Bellini