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Production of beauvericin by different races of *Fusarium oxysporum f. sp. melonis*, the Fusarium wilt agent of muskmelon

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Abstract

Fourty-four strains of *Fusarium oxysporum* were isolated from plants of melon with Fusarium wilt symptoms. Among these strains, thirty-nine were characterized for their pathogenicity on melon. Thirty-seven strains belonged to known races of *F. oxysporum f. sp. melonis*, while two strains were non-pathogenic. Four strains belonged to race 0, seven to race 1, four to race 2, and twenty-two to race 1,2. Beauvericin was produced by thirty-six strains in a range from 1 to 310 $\mu g\, g^{-1}$. Eight isolates of race 1,2 did not produce the toxin. In addition, of the two non-pathogenic strains, only one strain produced the toxin (290 $\mu g\, g^{-1}$). The production of enniatin A_1, enniatin B_1, and enniatin B was also investigated. Enniatin B was the only enniatin detected, being produced by eleven strains belonging to all the races, with a range of production from traces to 60 $\mu g\, g^{-1}$. Finally, melon fruits belonging to two different cultivars (*Cantalupo* and *Amarillo*) were artificially inoculated with one strain of *F. oxysporum f. sp. melonis* (ITEM 3464). Beauvericin was detected in the fruit tissues of both cultivars at a level of 11.2 and 73.8 $\mu g\, g^{-1}$, respectively. These data suggest that the production of both the toxins is not related to the pathogenicity of *F. oxysporum f. sp. melonis*, nor to the differential specificity of the races. The results confirm that beauvericin is a common metabolite of phytopathogenic *Fusarium* species.

Introduction

Beauvericin is a cyclodepsipeptide metabolite, closely related to the enniatins (Gaumann et al., 1960), a group of compounds with well known phytotoxic activity (Hermann et al., 1996; Burmeister and Plattner, 1987). Beauvericin was first reported as a compound produced by entomopathogenic fungi (Hamill et al., 1969; Bernardini et al., 1975; Gupta et al., 1991), and subsequently was shown to be produced by phytopathogenic fungi (Logrieco et al., 1993). In particular, beauvericin was found as a compound produced by strains of *Fusarium subglutinans* isolated from maize ears worldwide (Moretti et al., 1995). The role of beauvericin as an important secondary metabolite of phytopathogenic species of *Fusarium* was confirmed when production was detected in several strains of *F. proliferatum* isolated from different diseased host plants, including maize, asparagus, and date palm (Plattner and Nelson, 1994; Moretti et al., 1996; Abdalla et al., 2000). An investigation of the *Fusarium* genus, showed that various species produced beauvericin, including some strains of *F. oxysporum* from maize (Logrieco et al., 1998). Sagakuchi et al. (2000) provided the only available data on the phytotoxic effects of beauvericin by comparing it with other toxins (e.g. fusaric acid and fumonisin B_1). Although beauvericin did not cause any symptoms

on roots of the plants tested (melon, tomato, wheat, and barley), it showed the highest toxicity towards all the protoplasts of the plants tested, and in particular towards melon protoplasts at the lowest concentration tested (10^{-2} μM; Sagakuchi et al., 2000). These findings opened new fields of investigation on the role that this metabolite could play in the muskmelon wilt disease.

Fusarium oxysporum f. sp. melonis is the causal agent of Fusarium wilt of muskmelon (*Cucumis melo*). Based on the pathogenicity reaction of a set of differential cultivars within *C. melo*, this *forma specialis* has been divided in to four races: 0, 1, 2, and 1,2, that correspond to the resistance genes that are overcome (Risser et al., 1976). Race 1,2 has been further subdivided into two different pathotypes 1,2y (yellows) and 1,2w (wilt), based on the type of symptoms induced (Bouhot, 1981). Although host specialisation has been historically identified as the most important trait in *F. oxysporum* and has been used as the basis of classification, the occurrence of multiple races in *F. oxysporum f. sp. melonis* showing diversity in virulence led scientists to question whether pathogenicity is the most meaningful basis on which to classify sub-*formae speciales* groupings (Jacobson and Gordon, 1991). The identification of eight vegetative compatibility groups (VCGs) within *F. oxysporum f. sp. melonis* and the fact that all four races can be in more than one VCG, as well as all the races being present in a single VCG (Jacobson and Gordon, 1990), showed that no race constitutes a genetically homogeneous group (Jacobson and Gordon, 1991). Moreover, races were not distinguished by a significant intergenic spacer (IGS) of the rDNA (Appel and Gordon, 1996). The physiological, genetic, or molecular determinants that could be the basis of pathogenic race differentiation are still unclear. Therefore, on the basis of this knowledge, the main object of this work was to investigate the production of beauvericin and related compounds as possible sources of biochemical determinants of pathogenicity to provide a better understanding of the relationships within this *formae speciales* of *F. oxysporum* and to assess the possible role that these metabolites could play in Fusarium wilt of muskmelon. Moreover, infection by *F. oxysporum f. sp. melonis* can also develop on fruits both in the field and during storage (Brigati and Gori, 1986). Therefore, some melon fruits were inoculated with a strain of *F. oxysporum f. sp. melonis* and the beauvericin occurrence in the fruits studied, in order to obtain information on the ability of this fungus to produce the toxin *in vivo*.

Materials and methods

Fungal strains

All fourty-four strains were isolated from muskmelon plants with typical Fusarium wilt symptoms. Thirty-two strains of *F. oxysporum f. sp. melonis* were isolated in the main areas of muskmelon cultivation in Italy by A. Belisario and L. Corazza (Istituto Sperimentale per la Patologia Vegetale (ISPaVe), Rome) and twelve strains were provided by T.R. Gordon (University of Davis, California) (Table 1). Tissues of muskmelon with symptoms of disease, were surface-disinfected with 1% NaOCl for 10 min, rinsed in sterile distilled water (SDW) and directly placed on Petri dishes containing a modified pentachloronitrobenzene medium selective for *Fusarium* (Nelson et al., 1983). Single spores of putative *Fusarium* colonies were transferred to carnation leaf agar (Nelson et al., 1983) for morphological identification.

Pathogenicity test

Fourteen-day-old cultures of *F. oxysporum* isolated from melon grown at room temperature (20–25 °C), were flooded with SDW and scraped with a sterile glass rod to obtain a purple–pink spore suspension. This slurry was filtered through two layers of sterile cheesecloth and the filtrate diluted with SDW to obtain spores (a mixture of macroconidia and microconidia) at a concentration of 10^6 ml^{-1}. Seeds of melon were surface-disinfected with 1% NaOCl for 20 min, rinsed in SDW, and sown in cell-type plastic growing trays (Bamaplast, Pistoia, Italy), one seed per cell (10 cm diam), filled with an autoclaved potting mix of peat and sand (1 : 1, v/v).

The differential varieties of muskmelon used were: Charentais T which lacks any genes for resistance, Charentais *Fom-1* resistant to race 0 and race 2, Charentais *Fom-2* resistant to race 0 and race 1, and Margot which carries both the resistance genes *Fom-1* and *Fom-2*. Seedlings were inoculated at the cotyledon to first-true-leaf stage when plants were about 10 days old, as this stage of maturity was shown to be most susceptible to Fusarium wilt. Plants were removed from the seedling trays and the roots washed in tap water, pruned to a length of about 2.5 cm, and dipped at the same time for 1 min into the spore suspension. The inoculated seedlings were transplanted into

Table 1. Origin, race, and toxin production of strains of *F. oxysporum f. sp. melonis*

Isolate ITEM	Isolate ISPaVE	Origin	Race	BEA ($\mu g\,g^{-1}$)*	ENN-B ($\mu g\,g^{-1}$)
3451	1279	Southern Italy	0	115	n.d
3463	1413	California	0	2	10
3465	1409	California	0	20	10
3471	1405	California	0	105	60
3281	1069	Sicily	1	100	n.d
3285	1074	Sicily	1	10	n.d
3288	1077	Sicily	1	75	n.d
3452	1281	Southern Italy	1	30	n.d
3464	1404	California	1	80	n.d.
3467	1407	California	1	35	15
3472	1418	California	1	1	n.d
3455	1286	Sicily	2	135	40
3461	1411	California	2	40	n.d.
3462	1412	California	2	15	10
3468	1406	California	2	10	3
3470	1417	California	2	2	Traces
3278	1018	Central Italy	1,2	90	10
3279	1027	Sicily	1,2	5	5
3280	1068	Sicily	1,2	65	40
3282	1070	Sicily	1,2	15	n.d
3283	1071	Sicily	1,2	n.d	n.d
3284	1073	Sicily	1,2	40	n.d
3286	1075	Sicily	1,2	n.d	n.d
3287	1076	Sicily	1,2	n.d	n.d
3289	1078	Southern Italy	1,2	265	n.d
3290	1079	Sicily	1,2	60	n.d
3291	1080	Sicily	1,2	310	n.d
3292	1081	Sicily	1,2	60	n.d
3293	1083	Southern Italy	1,2	n.d	n.d
3294	1084	Southern Italy	1,2	20	n.d
3295	1086	Southern Italy	1,2	25	n.d
3296	1299	Central Italy	1,2	30	n.d
3297	1222	Central Italy	1,2	35	n.d
3453	1291	Sicily	1,2	n.d	n.d
3456	1287	Southern Italy	1,2	5	n.d
3466	1415	California	1,2	n.d	n.d
3469	1416	California	1,2	95	n.d
3298	1296	Sicily	Nonpathogenic	n.d	n.d
3450	1304	Sicily	Nonpathogenic	290	n.d
3299	1219	Central Italy	—	140	n.d
3454	1299	Southern Italy	—	150	n.d
3300	1289	Southern Italy	—	105	20
3459	1303	Southern Italy	—	50	n.d
3460	1283	Sicily	—	55	n.d

ITEM: Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy; ISPaVE: Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; BEA: beauvericin; ENN-B: enniatin B; n.d.: not detected; —: race not determined. * $\mu g\,g^{-1}$ of dried weight of fungal cultures.

plastic growing trays (one plant per cell) filled with a steamed potting mix (1 part each of soil : sand : peat) and placed in a greenhouse at 20/28 °C, night/day temperatures. The roots of control plants were pruned to a length of about 2.5 cm and dipped in tap water prior to transplanting. The number of dead and healthy plants was recorded 21 days after inoculation. Plants were rated as susceptible to Fusarium wilt if they died, or resistant to Fusarium wilt if they remained free of wilt symptoms.

In vitro *toxin production*

Single-conidium strains were grown on 10 g of autoclaved rice kernels adjusted to about 45% moisture in 50-ml Falcon tubes and inoculated with 2 ml of an aqueous suspension containing approximately 10^7 conidia ml^{-1}. Cultures were incubated at 25 °C for 4 weeks. The culture material was dried in a forced draft oven at 60 °C for 48 h, finely ground and stored at 4 °C. Controls were subjected to the same treatment but inoculated with SDW. Results were expressed at $\mu g\ g^{-1}$ dry weight.

In vivo *toxin production*

Melon fruits of two different cultivars (three for each cultivar), Cantalupo and Amarillo, were inoculated in the peduncle area with 2 ml of an aqueous suspension containing approximately 10^7 conidia ml^{-1}. The melon fruits were maintained at room temperature for one week. The fruit tissues were dried in a forced draft oven at 60 °C for 48 h, ground and stored at 4 °C. Control fruits were subjected to the same treatment without being inoculated.

Toxin analyses

Water for HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA). Organic solvents (HPLC grade) were purchased from Merck (Switzerland). The standards for beauvericin (catalogue no. B7510) and enniatin mixture (catalogue no. E3643) were purchased from Sigma Chemical Co. (St. Louis, MO). The enniatin mixture contained enniatin B – 19%, enniatin B_1 – 54%, enniatin A – 3%, and enniatin A_1 – 20%.

To extract BEA and enniatin, 5 g of each sample (of both melon and fungal cultures on rice) was ground and homogenized in a Ultraturax for 3 min with 25 ml of methanol (99.5%). Samples were filtered through Whatman no. 4 filter paper and methanol was removed under reduced pressure. Extracts were resuspended in 3 ml of methanol and pre-purified once on a C18 column (Varian Inc., Palo Alto, CA). The column was activated with 3 ml of methanol and the loaded extract eluted with 2 ml of methanol. The extract was concentrated to 1 ml and filtered through an Acrodisk filter (pore size, 0.22 μm) (Millipore, Jonezawa, Japan) before high-performance liquid chromatography (HPLC). Twenty microlitres of the extract were loaded onto the column. Beauvericin and enniatin (B, B_1, and A_1) analyses were performed according to Monti et al. (2000) with minor modifications. HPLC analyses were performed using LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Shiseido Capcell Pak C_{18} ($250 \times 4.6\ mm^2$, 5 μm) column was used. HPLC conditions included a constant flow at 1.5 ml min^{-1} and acetonitrile-water (65 : 35 v/v) as a starting eluent system. The starting ratio was kept constant for 5 min and then linearly modified to 70% acetonitrile in 10 min. After 1 min at 70% acetonitrile, the mobile phase was returned to the starting conditions in 4 min. Beauvericin and the enniatins were detected at 205 nm. Toxins were identified by comparing retention times and UV spectra of samples with authentic standards. Further confirmation was obtained by co-injecting standards with each sample. Toxins were quantified by comparing peak areas from samples to a calibration curve of standards. Chemical structures were confirmed with a liquid chromatography-mass spectrometer (LC-MS). HPLC conditions described above for the analytical separations were applied, but a different HPLC system was used. A Perkin–Elmer LC series 200 connected to a 785A UV/VIS detector was coupled with an API-100 single quadruple mass spectrometer (Perkin–Elmer Sciex Instruments, Ontario, Canada). A flow rate of 20 μl min^{-1} was split from the LC eluent into the ion spray source. A probe voltage of 5300 V and de-clustering potential of 50 V were used. Full-scan spectra were acquired from 400 to 800 amu using a step size of 0.5 amu and a dwell time of 4.2 ms. The instrument mass-to-charge ratio scale was calibrated with the ions of the ammonium adducts of polypropylene glycol. The detection limit was 20, 1.3, 3.6, and 1.2 $ng\ g^{-1}$ for beauvericin, enniatin A_1, enniatin B_1, and enniatin B, respectively.

Results

Data on race classification and toxin production are reported in Table 1. The pathogenicity assays, performed with thirty-nine strains, showed that all four races of the pathogen occurred among this set of isolates. In particular, four strains belonged to race 0, seven strains to race 1, four strains to race 2, and twenty-two to race 1,2; in addition, two non-pathogenic strains were also characterized. For toxin production, among the forty-four strains tested, thirty-six strains produced beauvericin in a range from 1 to 310 $\mu g\ g^{-1}$. In particular, the four strains of race 0 produced

beauvericin at 2–115 μg g^{-1}, the seven strains of race 1 produced the toxin from 1 to 100 μg g^{-1}, and all the four strains of race 2 produced beauvericin in a range from 10 to 135 μg g^{-1}. Among the twenty-two strains of race 1,2, only sixteen produced beauvericin, in the range 2–310 μg g^{-1}. In addition, of the two non-pathogenic strains, only one strain produced the toxin (290 μg g^{-1}). Five strains included in this study, isolated from diseased melon plants but not characterized for pathogenicity, produced beauvericin (from 50 to 150 μg g^{-1}). The production of enniatin A_1, enniatin B_1, and enniatin B was also investigated. Enniatin B was the only enniatin produced by these cultures and only by eleven of the cultures with a range of concentration from traces to 60 μg g^{-1} (Table 1).

Melon fruits inoculated with strain ITEM 3464 resulted in contamination with beauvericin. Cantalupo fruits contained 11.2 μg g^{-1} of the toxin (mean value), and Amarillo fruits contained 73.8 μg of beauvericin per gram.

Discussion

This is the first report on beauvericin production by strains belonging to a *forma specialis* of *F. oxysporum*. The data showed that no relationship was found between a specific race and the production of beauvericin, since isolates belonging to all four races were able to produce the toxin. These findings suggest that this metabolite is not related to specificity of the races of *F. oxysporum f. sp. melonis* and the level of production of beauvericin (Table 1) *in vitro* does not appear related to pathogenicity, since the two non-pathogenic isolates gave opposite results; ITEM 3459, can be considered as a high producer, while ITEM 3296 did not produce any detectable amount of toxin. On the other hand, Sagakuchi et al. (2000) reported that beauvericin was highly toxic to melon protoplasts compared with other toxins with known phytotoxic effects, such as fusaric acid and fumonisin B_1 (Desjardins and Hohn, 1997). Moreover, Lemmens et al. (2000), who investigated the mode of action of beauvericin towards biological membranes using artificial membranes as a model, reported that beauvericin is a very potent channel-forming molecule as it induces pores (which are selective for cations and non-permeable anions) even when present in trace amounts. It was concluded that the non-selective toxic activity of beauvericin could be explained by its ability to induce pores in biological membranes, resulting in a disturbance of the normal gradients of physiologically important monovalent cations across membranes (Lemmens et al., 2000). This mechanism could be expressed by beauvericin in melon plants, once the metabolite has passed through the cell wall of the melon and is in contact with the cell membrane. Such a process could determine a role for beauvericin in the pathogenicity caused by strains of *F. oxysporum f. sp. melonis* that produce the toxin.

Some of the strains also produced enniatin B, a toxin that has been shown to be phytotoxic (Burmeister and Plattner, 1987; Gaumann et al., 1960) and to have a specific role in plant pathogenesis (Herrmann et al., 1996). Although these depsipeptides do not seem to be related to the ability of pathogens to cause disease since they are produced by all races of the pathogen (Table 1), the ability of some strains to produce both the toxins could provide them with further tools for causing phytotoxic effects in melon plants. In addition, the occurrence of beauvericin in the melon fruits artificially inoculated by strain ITEM 3464 showed that the fungus could accumulate the toxin in the fruits. This is important, since melon fruits are especially sensitive to fungal attack during all stages from harvest to storage, as a result of mechanical damage during processing, which helps the fungus to penetrate the fruits through wounds around the peduncle (Brigati and Gori, 1986).

The ability of *F. oxysporum f. sp. melonis* strains to produce both beauvericin and enniatin B, besides confirming the importance of beauvericin as a common metabolite of phytopathogenic *Fusarium* species, suggests that further investigations on the possible additive or synergistic effects of both the toxins on melon plants should be carried out.

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