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Pablo Marcelo Yaryura [a], Gabriela Cordón [b], Mariana León [a], Norma Kerber [a], Norma Pucheu [a], María Gabriela Lagorio [b], Gerardo Rubio [a], Jorge Vivanco [c] & Augusto García [a]

[a] Facultad de Agronomía, UBA, Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA-CONICET), Buenos Aires, Argentina

[b] Facultad de Ciencias Exactas y Naturales, UBA, Química Inorgánica, Analítica y Química Física/INQUIMAE, Ciudad Universitaria Pab. II Piso 1, Buenos Aires, Argentina

[c] Center for Rhizosphere Biology, Colorado State University, Fort Collins, Colorado, USA

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[1]*Facultad de Agronomía, UBA, Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA-CONICET), Buenos Aires, Argentina*
[2]*Facultad de Ciencias Exactas y Naturales, UBA, Química Inorgánica, Analítica y Química Física/INQUIMAE, Ciudad Universitaria Pab. II Piso 1, Buenos Aires, Argentina*
[3]*Center for Rhizosphere Biology, Colorado State University, Fort Collins, Colorado, USA*

□ *Fluorescent compounds have been reported in plant leaves, having an important biological role in the protection towards UV irradiation. It is also known that stress conditions in plants stimulate the production of some of these compounds in their aerial portion. However, less is known about their production by roots or seeds and their presence in the soil surrounding them. We report here that, soybean, sunflowers and rapeseed produced fluorescent seed and root exudates. Phosphorus deficiency in rapeseed stimulates the excretion of fluorescent compounds by roots, mainly chlorogenic acid and scopoletin. When the effect of the fluorescent fraction from soybean seed exudates on* Macrophomina phaseolina *was tested, an enhanced induction of sclerotia formation, hyphal squeezing and lysis in the mycelium was observed. The fluorescent compounds and perhaps other chemical molecules also present in exudates might constitute a transitory defense barrier against early attack by pathogens.*

Keywords: mineral stress, fluorescent compounds, oil crops, *Macrophomina phaseolina*, phenolic compounds

INTRODUCTION

Secondary metabolites excreted by roots are highly diverse. Some of them, such as phenolic compounds like caffeic acid (CFA), p-coumaric acid

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Address correspondence to Pablo Yaryura, Facultad de Agronomía, UBA, Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA-CONICET), Av. San Martín 4453, C1417DSE, Ciudad Autónoma de Buenos Aires, Argentina. E-mail: yaryura@agro.uba.ar

(PC), ferulic acid (FA), chlorogenic acid (CGA), vanillic acid (VA), daidzein (DAI), and scopoletin (SC) are fluorescent. Although in most cases the real function of these fluorescent compounds is unknown, there has been some attempts to describe their involvement in plant functioning. Lang et al. (1991) suggested that the fluorescent compounds secreted by plant leaves act as a barrier against solar UV light thus protecting the cells of the mesophyll containing chlorophyll. In flowers of *Mirabilis jalapa*, Gandía-Herrero et al. (2005) observed that the visible fluorescence emitted by a yellow betaxanthin pigment is absorbed by another pigment, a violet betacyanin, to create a contrasting fluorescent pattern on the flower's petals. This modifies the way in which the flower appears to insects and could thus be crucial for pollinator reception.

There are some reports on the capacity of UV light to penetrate the superficial soil layer (Schober and Lohmannsroben, 2000), causing biological responses including changes in the exudates (Nagahashi and Douds, 2004; Zaller et al., 2002; Gamliel and Katan, 1992).

In Argentina, soybean, sunflower and rapeseed are included in the most common agricultural rotations either in single or double cropping with maize and wheat. Moreover, almost 70% of the agricultural soils are deficient in phosphorus (P), constituting one of the main constraints to crop yield. In this paper we studied the relationship between the deficiency of this nutrient and the fluorescent root excretion.

Macrophomina phaseolina is responsible for charcoal rot disease in more than 500 plant species (Sinclair, 1982) and is one of the most damaging soil-borne pathogens in our region, being able to infect the three species studied (Gaetán et al., 2006; Bressano et al., 2010; Hussain et al., 1990). In addition, we show here that this fungus was susceptible to fluorescent phenolic compounds and we chose it as a model system to work with.

With the aim of further increasing the knowledge on the possible role of fluorescent compounds exuded by plant roots, experiments were performed under sterile conditions in order to evaluate whether: i) crop plants secrete fluorescent root or seed compounds; ii) plant stress affects the secretion of fluorescent compounds by roots; and iii) the fluorescent compounds excreted by seeds or roots could help plants to resist early challenges by fungal species temporarily.

MATERIALS AND METHODS

Plant Growth Conditions and Preparation of Root and Seed Exudates

Seeds of soybean (*Glycine max* L.) 'Ferias del Norte' ('FN-4.85'), sunflower (*Helianthus annuus,* variety 'Paraiso 30', Nidera, Rotterdam, The Netherlands) and rapeseed (*Brassica napus*, variety 'Filial precoz', Bioproductos, St. Thomas, Virgin Islands) were surface-disinfected by soaking in

3% sodium hypochlorite (NaOCl) for 10 min and rinsed three times with sterile distilled water.

To collect root exudates, disinfected and pre-germinated seeds (2 days at 25°C in the dark) were transferred to glass tubes containing 20 mL of a sterile mineral solution (MS) as described by Murashige and Skoog (1962), either with 1.25 mM potassium dihydrogen phosphate (sufficient amount of phosphate to sustain a normal growth), (+P) or without phosphate (−P). Potassium concentration was kept at a constant level by the addition of potassium chloride (KCl), maintaining the original pH of the MS (pH: 5.8). A filter paper (Whatman 3 MM) with an inverted U shape was previously inserted into the glass tube as mechanical support of the seedlings. Plants were grown in an orbital shaker at 140 rpm, placed in a plant growth chamber and kept at a 16 h light-8 h dark cycle, in which light was emitted at a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} using Philips TLD 865 fluorescent tubes (Philips, Eindhoven, The Netherlands). Temperature was kept constant at 22 ± 2°C and relative humidity, RH (±10%) was 70% during the day and 90% during the night. The experiment involved three crop species, two P levels (with or without P), 10 replicates per species and level of P and at least three independent series of experiments. To maintain the initial medium volume, half strength of MS medium was added with or without phosphate according to each case, every week and the pH was adjusted to 5.8.

Plants were harvested 40 days after transplanting. At this time, plants were removed from the glass tubes and root exudates collected from the remaining growing solution. The final solution was filtered under sterile conditions to remove sloughed root caps or border cells, and finally lyophilized.

To collect seed exudates, 10 g of surface-disinfected seeds of the three plant species were transferred into 500 mL Erlenmeyer flasks containing 100 mL of sterile distilled water and incubated in continuous shaking (140 rpm) at 30°C. Seeds were kept for 20 h after the beginning of the germination process. The corresponding exudates were filtered under sterile conditions and lyophilized.

To verify if both root and seed exudates remained sterile throughout their collection, samples obtained before sterile filtration were incubated in potato dextrose agar (PDA) and nutrient agar (NA).

Determination of Shoot and Root Dry Weight

Total plant, and also shoot and root dry weights were determined after drying the plants at 60°C until constant weight.

Fluorescence Measurements

Fluorescence spectra were recorded for seed and root exudates from each of the three plant species. In the case of root exudates, the plants

were grown either in the presence or the absence of phosphate. For seed exudates of each of the three plants, fluorescence spectra were recorded between 380 and 600 nm, with excitation wavelength at 360 nm. For soybean root exudates, fluorescence spectra were recorded between 350 and 600 nm, with excitation wavelength at 340 nm, while for rapeseed and sunflower root exudates fluorescence spectra were recorded between 320 and 580 nm, with excitation wavelength at 300 nm.

The excitation wavelength was selected in each case, based on the maximum in the respective excitation spectra (not shown), thus ensuring the attainment of maximum possible fluorescence intensity. A steady-state spectrofluorometer was used in all cases (Quanta Master, PTI -Photon Technology International, Brunswick, NJ, USA). Fluorescence spectra were obtained as the number of counts recorded by the spectrofluorometer photomultiplier (810, PTI -Photon Technology International) as a function of wavelength.

Extraction of Phenolic Compounds from Soybean Seed Exudates

Method 1

10 mL of lyophilized soybean seed exudate was extracted twice with 5 mL each of 80% (v/v) methanol-water and then homogenized for 1 min and centrifuged for 10 min at 10,000 rpm. The supernatant fractions were combined, lyophilized and finally re-suspended in 1 mL of 80% (v/v) methanol-water. The pellet was dried with argon and re-suspended in 1 mL of distilled water (de Ascensao and Dubery 2003). The fluorescence spectrum was recorded in both the organic and aqueous phases.

Method 2

The first part of this method, which is the extraction with 80% (v/v) methanol-water, was identical to that described for method 1. In this case, the pellet, dried with argon, was re-suspended in 5 mL of 2 M sodium hydroxide (NaOH), shaken for 1 h in the dark and brought to pH 6.0 with 12 N hydrochloric acid (HCl). Thereafter, this fraction was extracted twice with 5 mL each of ethyl acetate. The last two fractions were combined, evaporated and re-suspended in 1 mL of 80% methanol-water (Kováčová and Malinová, 2007). The aqueous phase was lyophilized and finally re-suspended in 1 mL of distilled water. The fluorescence spectrum was recorded in the two organic phases and in the aqueous one.

Method 3

Ten mL of lyophilized soybean seed exudate was extracted twice with 2 mL of 1% (v/v) of HCl in methanol; the two extractions were combined and left stand without shaking for 1.5 h. After centrifuging for 10 min at

10,000 rpm, the supernatant fractions were combined, lyophilized and finally re-suspended in 1 mL of 80% (v/v) methanol-water. The pellet was dried with argon and re-suspended in 1 mL of distilled water (Cheel et al., 2005). The fluorescence spectrum was recorded in both the organic and aqueous phases.

Method 4

Ten mL of lyophilized soybean seed exudate was re-suspended in 5 mL of 2 M NaOH, shaken for 1 h in the dark and brought to pH 2.0 with 6N HCl-water. The acid solution was extracted twice with 5 mL of chloroform. These two fractions were combined, evaporated and re-suspended in 1 mL of 80% methanol-water (Sun et al., 2001). The aqueous phase was lyophilized and finally re-suspended in 1 mL of distilled water. The fluorescence spectrum was recorded in both the organic and aqueous phases. The only fluorescent aqueous phase obtained (M4) was lyophilized and finally re-suspended in 1 mL of distilled water and dialyzed against 1 L of distilled water at 10°C overnight, using a dialysis bag (Sigma Aldrich, D2272; Sigma Aldrich, St. Louis, MO, USA) retaining molecules of MW < 2.0 kD. The fraction outside the dialysis bag was lyophilized and re-suspended in 1.0 mL of distilled water. The fluorescence spectra were recorded for aqueous phases, both inside and outside the dialysis bag.

Identification of Phenolic Compounds by HPLC

Lyophilized root and seed exudates (10 mL of each) were extracted twice with 5 mL each of 80% (v/v) methanol-water and then homogenized for 1 min and centrifuged for 10 min at 10,000 rpm. The supernatant fractions were combined and filtered through a 0.2 μm filter prior to analysis. All standards were prepared by dissolving the pure compounds purchased from Sigma Chemical Co. in 80% (v/v) methanol-water.

For HPLC experiments, a Shimadzu LC-9A model instrument (Shimadzu Corp., Tokyo, Japan) with a fluorescent detector was used, setting the optimum at 320 nm, which was the wavelength used for detection and quantification of the phenolic compounds. The column used was a Phenomenex Luna C_{18} (5 μm, 250 mm × 4.6 mm; Phenomenex Luna, Torrance, CA, USA) and column temperature was kept at 25°C. An aliquot of 25 μL of either the standards or the samples was injected into the HPLC system. The elution was performed using a linear gradient between 10 to 100% of solvent B in A (solvent A: acetic acid/water (2:98 v/v) and solvent B: acetic acid/acetonitrile/water (2:30:68 v/v/v) as mobile phase). Phenolic compounds from the exudate samples were identified by co-elution with the following standards: CFA, FA, CGA, PC, DAI and SC. During HPLC analysis the solvent gradient was run for 30 min with a flow rate of 1.2 mL min^{-1}. All

the standards and solvents used were of analytical and HPLC grade. These analyses were performed at the Analytix Laboratories, Manhattan, KS, USA.

Growth of *M. phaseolina*

M. phaseolina was grown in malt extract agar (MA) (20 g l^{-1} malt extract, 2 g L^{-1} meat peptone, 20 g L^{-1} glucose, 15 g L^{-1} agar) at 30°C for five days.

Determination of the Effect of Aqueous and Organic Extracts of Soybean Seed Exudates on the Growth of *M. phaseolina*

Two opposite circular wells, each located in the periphery of MA plates, were filled with the different fractions to be tested, placing 80% methanol-water (solvent used to re-suspend all organic fraction obtained with the extraction methods mentioned before) in one well as a control. A 5 mm disc of young *M. phaseolina* mycelium was placed in the center of the plate. Also, pure fluorescent compounds (CFA, FA, CGA, SC, PC, DAI and VA) were tested. The plates were incubated at 25°C for five to seven days.

Statistical Analysis

All experiments were performed at least three times, and the results were analyzed by one-way analysis of variance (ANOVA) using the SAS software (SAS Institute Inc., Cary, NC, USA) and means were compared by the Tukey's test ($P < 0.05$).

RESULTS

Phosphorus Deficiency and Biomass of Crop Plants

The effects of P deficiency on root and shoot dry weight of soybean, sunflower, and rapeseed are shown in Table 1. Phosphorus stress induced an increase in the root to shoot ratio of the three plant species, although in a different proportion: 28, 43, and 500 % in soybean, sunflower and rapeseed, respectively. Rapeseed biomass production was the most affected by P stress: total biomass was reduced by 51% and aerial biomass by 64%. Soybean plants grown under P deficiency accumulated 12% less total biomass and 14% less aerial biomass than their counterparts grown in the presence of P. Neither total nor aerial biomass of sunflower plants showed significant differences when compared with plants grown in the presence of P.

Fluorescence of Root and Seed Exudates

The spectral characteristics of UV-induced fluorescence of root exudates affected by P stress were analyzed for the three plant species. The maximum

TABLE 1 Dry weight (mg) of soybean, sunflower and rapeseed plants, grown in the presence or absence of P

	Species					
	Soybean		Sunflower		Rapeseed	
	+P (mg)	−P (mg)	+P (mg)	−P (mg)	+P (mg)	−P (mg)
Total biomass	184.21 ± 5.83	161.83 ± 3.41*	84.07 ± 2.42	84.75 ± 4.0	65.10 ± 2.50	31.90 ± 2.90**
Root	27.73 ± 1.70	30.87 ± 1.38	10.25 ± 0.99	13.73 ± 0.88*	4.80 ± 0.32	10.45 ± 0.28***
Shoot	157.33 ± 5.67	134.63 ± 2.31**	73.82 ± 1.86	71.02 ± 3.55	60.50 ± 2.84	21.65 ± 2.65**
Root/shoot	0.18 ± 0.01	0.23 ± 0.01*	0.14 ± 0.01	0.20 ± 0.01*	0.08 ± 0.01	0.48 ± 0.05**

All data represent the mean ± standard error (n = 10). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Tukey's test). +P: control plants, grown in the presence of P, and −P: plants grown in the absence of P.

fluorescence of the root fluorescent compounds of the three plant species was verified at the same wavelength (410 nm) (Figure 1). In concomitance with that shown in Table 1, which shows the growth characteristics of the plants, rapeseed was the species most responsive to P stress. Thus, P deficiency induced an increase in the fluorescence of root exudates (Figure 1C), whereas soybean and sunflower were less affected (Figure 1A and 1B). When the fluorescence of root exudates was normalized considering plant-dry weight (inserts Figure 1A, 1B, and 1C, two right bars), only rapeseed showed a significant effect of P stress (insert 1C). In contrast, if fluorescence was normalized using root-dry weight, no differences were obtained for either growth condition (inserts Figure 1A, 1B and 1C, two left bars).

Germinating seeds of the three plants also excreted fluorescent compounds but with a maximum emission at 445 nm, and in this case, soybean seed exudates showed the maximum fluorescence intensity (Figure 2).

The presence of some phenolic compounds were analyzed, detected in aerial part of plants by Makoi and Ndakidemi (2007), in the exudates of the three plant species studied. The fluorescence spectra of the pure compounds such as CGA, SC, CFA, FA and PC, are all centered in a spectral region partially superimposed to that observed for the root and seed exudates (data not sown). HPLC-analysis of root exudates (Table 2) showed that CGA and SC increased when P deficiency was induced in rapeseed plants. While the fluorescent compounds were the same in the three plants investigated, the amounts of chlorogenic acid were different, being much lower in soybean and sunflower. In addition, no differences were detected in these last two species between the presence and absence of P.

Effect of Fluorescent Compounds of Root and Seed Exudates on *Macrophomina phaseolina*

To investigate whether phenolic compounds of seed and/or root exudates indeed exert a deleterious effect on fungal development, we measured

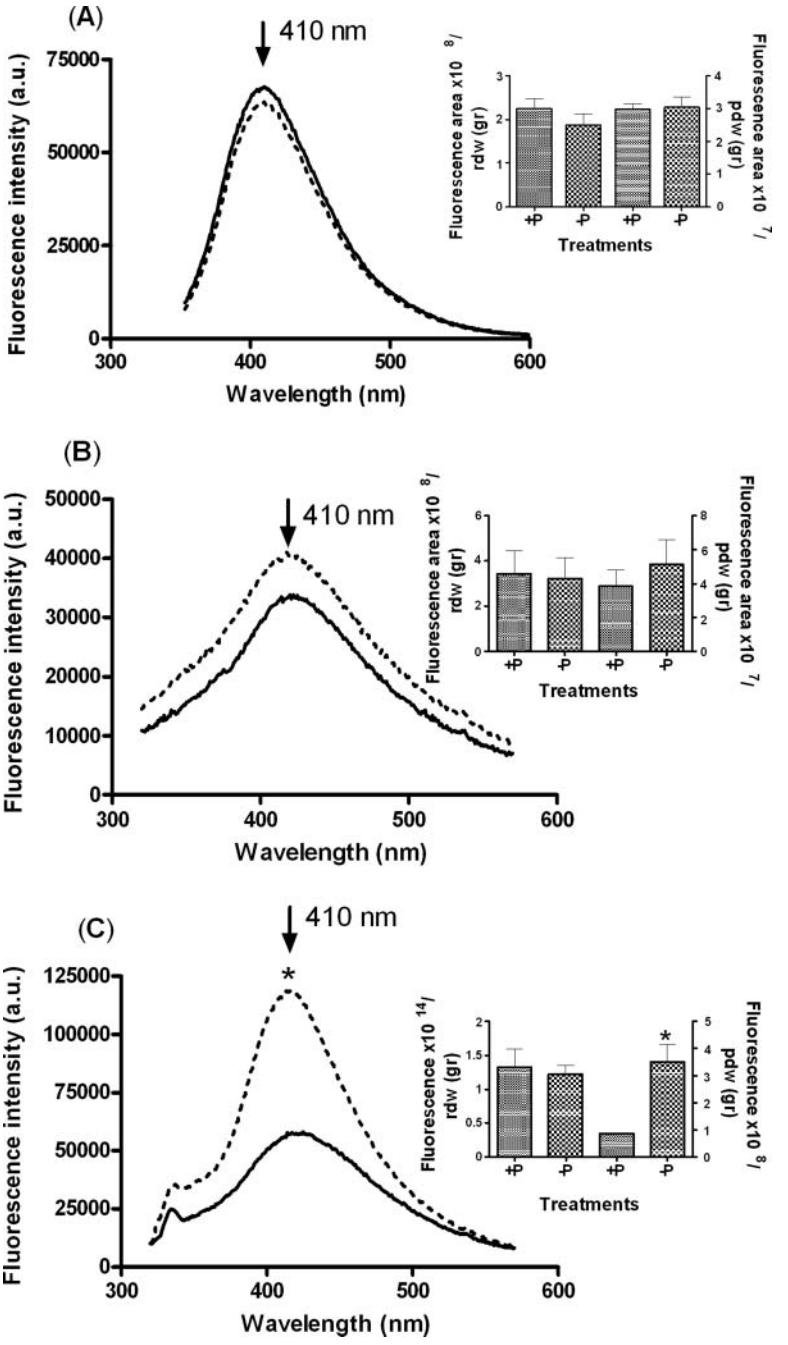

FIGURE 1 Average fluorescence emission spectra for root exudates of A) soybean, B) sunflower and C) rapeseed, both P-stressed (—) and non-stressed plants (—). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Tukey's test). a.u.: arbitrary units. Inserts: in each case the two right bars represent the fluorescence emission per g dry weight of stressed (−P) and non-stressed (+P) whole plants (pdw: plant dry weight) and the two left bars represent the fluorescent emission per g of the corresponding root dry weight (rdw), values are the mean of 10 replicates ±SE.

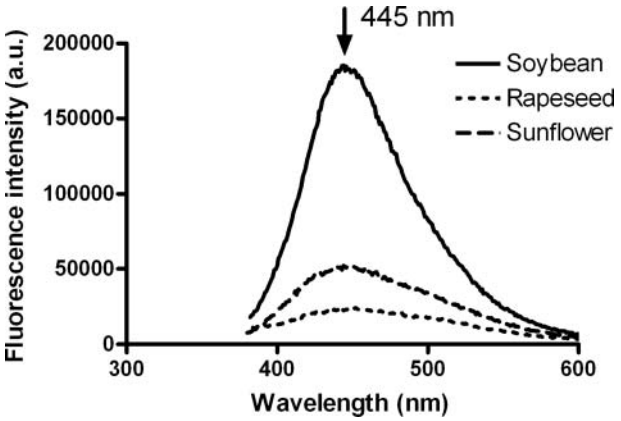

FIGURE 2 Average fluorescence emission spectra for seed exudates of soybean, sunflower and rapeseed. a.u.: arbitrary units. Data show fluorescence for exudates collected in an identical volume corresponding to identical seed weights.

their effect on *M. phaseolina* in *in vitro* experiments. As shown in Figure 2 the comparison of fluorescence intensity of seed exudates collected from the three species under comparable conditions revealed that soybean seeds exhibited the highest level of emission. On the other hands, in preliminary studies we detected that all root exudates had minor effect on *M. phaseolina* growth, and we, therefore, worked on soybean seed exudates to study those effects.

In order to concentrate the fluorescent compounds in the soybean seed exudates used, we devised a procedure based on their fractionation by means of organic solvents, resulting in an aqueous non-fluorescent phase and a fluorescent organic phase (M1, M2 and M3). In the particular case of M4, a fluorescent aqueous phase and a non-fluorescent organic phase (Figure 3 A, B) were produced. The fluorescence spectra of the organic fractions was centered in the range 435–450 nm, the difference being probably caused by

TABLE 2 Determination of phenolic compounds by HPLC.

Root exudates	Phenolic compounds (pmoles mL^{-1})		
	Chlorogenic acid	Scopoletin	Caffeic acid
Rapeseed (−P)	141.0 ± 3.5**	58.2 ± 4.0	28.4 ± 0.2
Rapeseed (+P)	63.2 ± 4.0	nd	21.6 ± 0.9
Sunflower (−P)	72.0 ± 2.6	60.0 ± 2.0	23.0 ± 1.0
Sunflower (+P)	64.9 ± 5.8	51.1 ± 1.6	20.0 ± 1.4
Soybean (−P)	21.0 ± 8.0	nd	27.6 ± 1.4
Soybean (+P)	18.5 ± 1.0	nd	24.6 ± 1.1

All data represent the mean ± Standard error (n = 5). $^{*}P < 0.05$; **P < 0.01; $^{***}P < 0.001$ (Tukey's test).

+P: control plants, grown in the presence of P, and −P: plants grown in the absence of P. nd: not detected.

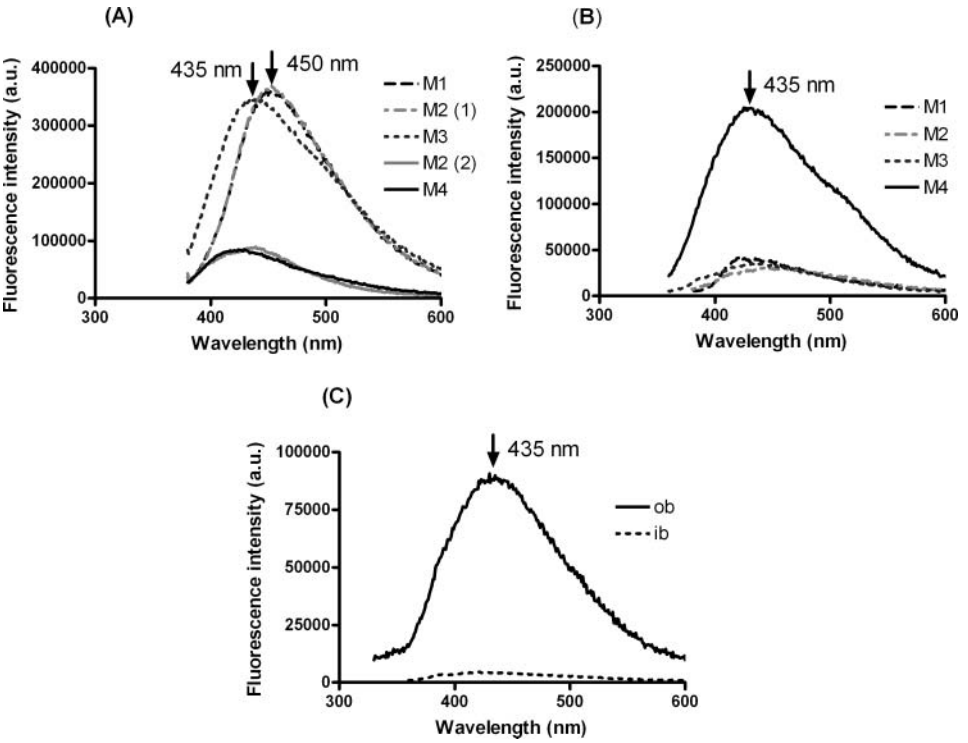

FIGURE 3 Fluorescence emission spectra of organic and aqueous phases obtained by fractionation of soybean seed exudates extracted by four different methods (M1, M2, M3 and M4 as described under Materials and Methods). A) organic phases; B) aqueous phases; C) dialysis of fluorescent aqueous phase obtained from M4. ob and ib represent the outside and the inside liquids of the dialysis bag respectively. M2(1) and M2(2) represent two different organic fractions of M2. a.u.: arbitrary units.

the solvent, while in the aqueous phases the maximum was at 435 nm. In the latter, the dialysis of the aqueous fluorescent fraction reflected the low MW (less than 2 kD) of the fluorescent compounds extracted (Figure 3C).

Figure 4B shows that only the fluorescent fractions derived from soybean seed exudates induced, in *M. phaseolina,* a significantly higher concentration of sclerotia than the control (Figure 4A). Also damages such as hyphal squeezing (Figure 4C) and lysis of the mycelia (Figure 4D) were observed. These effects, however, affected fungal growth as long as the external challenge with the exudate was kept. Growth reverted to normal upon transfer to a new plate without exudate. A similar but diminished effect was also caused by root and seed exudates of the other plant species and also by pure phenolic compounds (data not shown).

DISCUSSION

In nutrient-rich environments, a small root system is sufficient to satisfy the plant nutrient requirements because the high nutrient availability

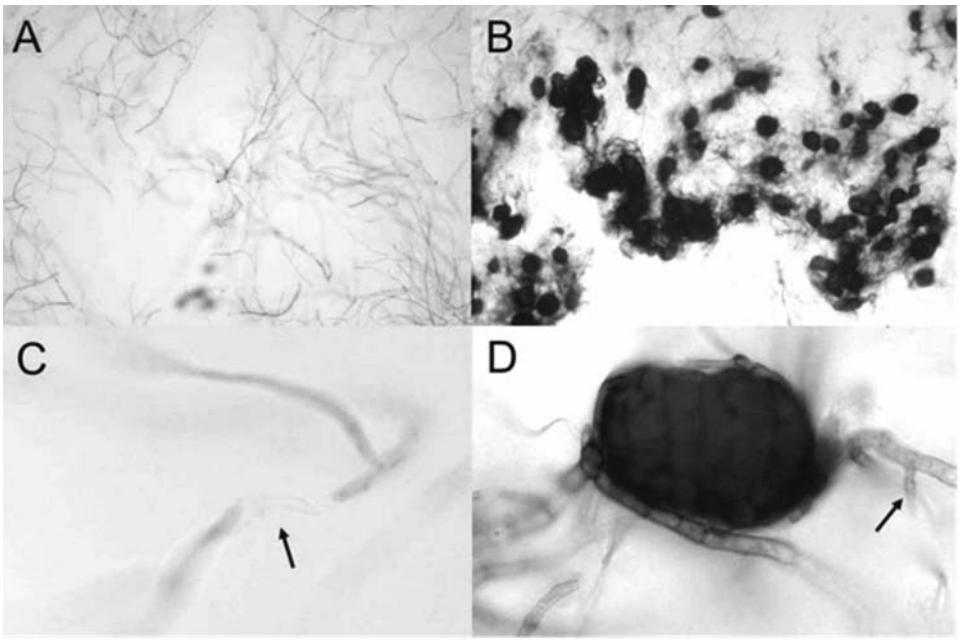

FIGURE 4 Microscopic observation of the effect of fluorescent extracts on *M. phaseolina*. A) normal hyphae (10x), taken from the control plate. B) Abnormally high number of sclerotia in the well with the fluorescent extract (10x). C) The arrow indicates hyphal squeezing (40x). D) The arrow indicates lysis of the mycelia (40x).

compensates for the lesser investment in root biomass (Bazzaz, 1997). Conversely, under low nutrient supply, plants usually increase the proportion of biomass allocated below ground. A greater proportion of roots would allow plants to capture more of the limiting resource (P in this case) and partly overcome the limitation (Lambers et al., 2006). The present results support these predictions since the three species studied showed consistent increases in the root/shoot ratio when growing under P stress conditions. Only rapeseed showed consistently less accumulation of biomass in response to the imposed P deficiency. The lower effect of P stress on growth of soybean and sunflower can be related to the seed size (rapeseed<sunflower<soybean), which limits the amount of P reserves contained in the seed (Atkinson, 1973). Rapeseed seeds are noticeably smaller than soybean and sunflower seeds, which mean that their P reserves are lower. Thus, rapeseed would experience P shortages in the surrounding environment earlier than the other two species, which can explain the responses of the studied species to the P treatments. This is probably why although a tendency to an increase in fluorescent secretion was observed in sunflower plants; the values were not significantly different between treatments.

Fluorescent compounds have been found in both above- and below-ground organs such as flowers, leaves and seeds (Gandía-Herrero et al., 2005;

Lang et al., 1991; Delannay and Palmer, 1982). Here, we observed that the three species consistently secreted fluorescent root exudates and unpublished experiments performed in our lab also indicated their widespread presence being also present as exudates in other crop species, such as wheat and maize.

Some authors have shown that there are stress conditions affecting the excretion of some phenylpropanoids by roots (Dixon and Paiva, 1995; Makoi and Ndakidemi, 2007). We observed that P stress caused a significant increase in the excretion of fluorescent compounds by rapeseed roots. These fluorescent compounds could be part of the phenylpropanoid biosynthetic pathway, some of them detected in the aerial part of plants (Makoi and Ndakidemi, 2007). Dixon and Paiva (1995) have shown that metabolic conversion of shikimic acid to CGA was preferentially induced by stress conditions, which could explain the increase, mainly in CGA, detected in our case. We have previously observed that rapeseed plants respond to P deficiency by increasing the concentration of anthocyanines in cotyledons and leaves, constituting a clear early sign of stress (Yaryura et al., 2009). This compound is also the result of shikimic acid metabolism (Dixon and Paiva, 1995).

On the other hand, it is interesting to note that fluorescent root and seed exudates induced sclerotia formation, hyphal squeezing and lysis in the mycelium in *M. phaseolina*, similar to those mentioned by Singh et al. (2008) when challenging this same fungus with *Bacillus subtilis* BN1. Interestingly we found that only the fluorescent fraction obtained from the extraction methods used affect the growth of *M. phaseolina*. When some pure phenolic compounds were tested, it was observed that sclerotia formation was induced, although the effect was less intense than that caused by fluorescent exudates at concentrations reasonably higher than those shown in Table 2, a fact that could indicate the importance of studying the whole or extracted exudate. The excretion of phenolic metabolites increased in plants under stress, which are supposed to be more susceptible to infection by fungi. This phenomenon appears as a transitory plant protection, since the increase in sclerotia formation distracts energy which could be otherwise used for myceliar development.

It is then possible to hypothesize that the phenolic compounds transitorily protect the plants under nutritional stress by diminishing the number of free mycelia, and that this condition is kept as long as phenolics are still being excreted.

Our results consistently showed that the presence of fluorescent root exudates is a ubiquitous characteristic of three economically relevant species studies. Regarding the role of these compounds in plant functioning, our results with *M. phaseolina* suggest that they may possess fungistatic activity. Whether this effect is related to the fluorescence properties of the specific compounds tested is difficult to determine, although analyzing this in further studies seems an interesting challenge. However, it is possible that the

structure of double bonds present in all fluorescent compounds, such as furanocoumarins, may, in some cases, cause phototoxic reactions (Asthana et al., 1993). Thus, the fluorescent compounds and perhaps other chemical molecules present in exudates might indeed constitute a transitory defense barrier against early attack by pathogens and microorganisms.

In conclusion, we can say that an increase in fluorescence of root exudates may be used as a general indication of the increased early stress conditions modeled here inducing a general P deficiency. This is best observed in rapeseed compared to either soybean or sunflower plants probably because in those latter, cotyledons provide the germinating plants with sufficient amounts of P.

Fluorescent compounds also have a fungistatic effect that is proportional to the amounts of fluorescent compounds excreted. In the present case, soybean seeds have both maximum fungistatic effect and the highest rate of production of fluorescent compounds. Rapeseed and sunflower have lesser fungistatic effects and concomitantly lesser rates of fluorescent compounds excretion.

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