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Characterization of strigolactones produced by *Orobanche foetida* and *Orobanche crenata* resistant faba bean (*Vicia faba* L.) genotypes and effects of phosphorous, nitrogen, and potassium deficiencies on strigolactone production

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ABSTRACT

In the present study, characterization of strigolactones (SLs) produced by some Tunisian faba bean genotypes partially resistant to *Orobanche foetida* Poir. and *O. crenata* Forsk. was conducted by LC–MS/MS and the results were compared with that of a susceptible genotype. Among the eight partially resistant genotypes grown hydroponically, only the genotype G5 and the susceptible one G9 (Badï) exuded into growth media similar mixture of three SLs, orobanchol, orobanchyl acetate, and a novel SL at amounts detectable by LC–MS/MS. This implies that, for the resistant genotypes except for G5, impaired SL production confers resistance to *Orobanche*. The amounts of orobanchol and orobanchyl acetate exuded by G5 and G9 were quantified by LC–MS/MS. Results showed that the susceptible genotype G9 produced larger amount of orobanchol than did the resistant genotype G5, and in both genotypes the amounts of orobanchol were significantly higher than that of orobanchyl acetate. Other mechanisms, acting after induction of *Orobanche* seeds germination, could be implied in the resistance of genotype G5. Since nutrient availabilities have been shown to affect SL production, effects of N, P and K deficiencies on SL production were studied for genotypes G5 and G9. Both N and P deficiencies enhanced SL exudation in both genotypes, while K deficiency did not affect it.

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1. Introduction

Orobanche foetida Poir. and *O. crenata* Forsk. are root holoparasites widely distributed in the Mediterranean area and inflicting severe damages on faba bean (*Vicia faba* L.) production. Among different control strategies, breeding of *Orobanche* resistant cultivars appears to be the most promising approach (Rubiales et al., 2006). However, resistance against broomrape in legumes is scarce, of complex nature and of low heritability, and these factors hamper the breeding of resistant cultivars (Rubiales et al., 2014). Under these circumstances, understanding mechanisms of naturally occurring resistance is expected to provide possible targets for breeding.

Orobanche seeds remain dormant in the soil for many years until they perceive germination stimulants released from host roots. Among known germination stimulants, strigolactones (SLs) are the most active stimulants inducing germination at as low as 10^{-12} M (Xie et al., 2010). More than 20 natural SLs have been characterized and novel ones are being identified (Yoneyama et al., 2013a).

Since seeds of root parasitic weeds need chemical stimuli including SLs for germination, reduced production and exudation of germination stimulants is a good trait for *Orobanche* resistance (Yoder and Scholes, 2010). Low induction of *Orobanche* seed germination by host-root exudates was reported in various legumes species including faba bean, vetch, pea, chickpea, and grass pea (Rubiales et al., 2003; Sillero et al., 2005; Pérez-de-Luque et al., 2005; Fernández-Aparicio et al., 2012), sunflower (Labrousse et al., 2001, 2004) and tomato (Dor et al., 2011). Fernández-Aparicio et al. (2014) reported that low SL production appeared to confer resistance against *Orobanche* observed in some resistant faba bean cultivars. However, impaired SL production may have pleiotropic effects on plant growth and development (Kohlen et al., 2011). In addition to their role as germination stimulants for root parasitic weeds, SLs function as a novel class of plant hormones regulating shoot and root architectures (Gomez-Roldan et al., 2008; Umehara et al., 2008; Kapulnik et al., 2011; Ruyter-Spira et al., 2011), photomorphogenesis (Shen et al., 2007), secondary growth (Agusti et al., 2011) and leaf senescence (Snowden et al., 2005; Yamada et al., 2014). SLs

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have been shown to be essential for root colonization by arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005; Besserer et al., 2006) and enhance rhizobia root colonization in pea and alfalfa (Soto et al., 2010; Foo and Davies, 2011) but may be inhibitory at higher concentrations as reported for *Medicago truncatula* (De Cuyper et al., 2014).

The objective of the present study is to identify resistance mechanisms of seven faba bean genotypes G1–G7 which have been recently characterized as *O. foetida and O. crenata* resistant in the fields at Beja and Ariana in Tunisia (Trabelsi et al., 2015). Najeh (G8) and Badï (G9) were included as resistant and susceptible checks, respectively. SLs produced by the faba bean genotypes were identified and quantified in order to examine if observed resistance is related to reduced SL exudation. Moreover, because nutrient availabilities have been shown to profoundly affect SL production (Yoneyama et al., 2007a, 2007b, 2012, 2013b; López-Ráez et al., 2008), effects of N, P, and K deficiencies on SL productions in selected genotypes of faba bean were also studied.

2. Materials and Methods

2.1. Plant material

Eight faba bean (*Vicia faba* L.) genotypes partially resistant to both *O. foetida* and *O. crenata* including a released variety Najeh (G1, G2, G3, G4, G5, G6, G7 and Najeh) and a susceptible check Badï (G9) were used in the experiments (Table 1). Seeds of the nine genotypes were harvested in 2011 from single selected plants grown separately under insect-proof cages at Beja experimental station in the North-West of Tunisia.

Seeds of *O. foetida* and *O. crenata* were collected from the parasites on faba bean during the cropping season 2010–2011, respectively from Beja and Ariana regions in Tunisia. *O. minor* seeds were collected from the parasites on red clover plants in Utsunomiya, Japan.

2.2. Hydroponic culture of faba bean

Faba bean seeds were surface sterilized in sodium hypochlorite (1.0%) containing 0.1% Tween-20 for 10 min and rinsed thoroughly with sterile Milli-Q water. The seeds were soaked in Milli-Q water at room temperature for 2 h and then sown in pots containing sterilized sand and placed in a growth chamber for 7 d with a16h photoperiod (120 μmol $m^{-2}.s^{-1}$) at 22 °C. Three healthy seedlings from each line were selected and transferred to a stainless steel sieve lined with a sheet of gauze moistened by placing it on the cup (9.5 cm in diameter, 17 cm deep, approx. 550 mL in volume) containing 350 mL of tap water. The plants were grown for 20 d during which the tap water growth media were replaced with fresh one every 2 d. Experiment was repeated three times.

2.3. Extraction of root exudates

The collected tap water growth media containing root exudates (approx. 300 mL + washing) were extracted three times with 100 mL of ethyl acetate. The ethyl acetate extracts were combined, washed with 0.2 M K_2HPO_4 (pH 8.3), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The crude extracts were kept at 4 °C until use.

2.4. Identification of strigolactones by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

High performance liquid chromatography (HPLC) separation was conducted with a U980 HPLC instrument (Jasco, Tokyo, Japan) fitted with an ODS (C_{18}) column (Mightysil RP-18, 2 × 250 nm, 5 μm: Kanto Chemicals Co., Ltd., Tokyo, Japan). The crude extracts were dissolved in 60% methanol and filtered through spin columns (Ultra-fee MC, 0.45 μm pore size; Millipore, Tokyo, Japan), and 3 μL was injected. The mobile phase was 60% methanol in water and was changed to 100% methanol 30 min after injection. The column was then washed with 100% methanol for 20 min. The flow rate was 0.2 mL min^{-1} and the column temperature was set to 40 °C.

Mass spectrometry was performed with a Quattro LC mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray source. The drying and nebulizing gas was nitrogen generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). The nebulizer gas flow was set to approx. 100 Lh^{-1}, and the desolvation gas flow to 500 Lh^{-1}. The interface temperature was set to 400 °C and the source temperature to 150 °C. The capillary and cone voltages were adjusted to orobanchol and the positive ionization mode. MS/MS experiments were conducted using argon as the collision gas and the collision energy was set to 16 eV. The collision gas pressure was 0.15 Pa. Known strigolactones were detected by using multiple reactions monitoring (MRM).

2.5. Quantification of strigolactones

Among the nine faba bean genotypes studied, only G5 and the susceptible check Badï (G9) were found to produce relatively large amounts of SLs (orobanchol, orobanchyl acetate, and a novel SL) and these genotypes were subjected to SL quantification. The plants were grown hydroponically and growth media (tap water) were collected as described before (Yoneyama et al., 2013a, 2013b). For quantification of orobanchol and orobanchyl acetate, [6′-^{2}H] orobanchol (400 pg) and [6′-^{2}H] orobanchyl acetate (200 pg) were added as internal standards to the collected growth media prior to solvent extraction. Crude root extracts obtained as described before were analyzed by LC–MS/MS using 5-channel MRM. The transitions of *m/z* 369–272, 370–272, 411–254, 412–255, and 427–270 were monitored for orobanchol, orobanchol-d_1,

Table 1
Pedigree, origin and main characteristics of faba bean genotypes used in the study.

Genotypes/Pedigree	Origin/characteristics
G1: XAR-VF00.12–12–3-1-3-1	Cross performed in Ariana (Tunisia) in 2000 between Tunisian breeding line resistant to *O. foetida* and large seeds population "Malti".
G2: XAR-VF00.13–8–3-1-1-1-1	Cross performed in Ariana (Tunisia) in 2000 between Tunisian breeding line resistant to *O. foetida* and faba bean small seeds breeding lines selected by INRA Rennes (France)
G3: XAR-VF00.13–89–2-1-1-1-1	Cross performed in Ariana (Tunisia) in 2000, between Tunisian breeding line resistant to *O. foetida* and faba bean small seeds breeding lines selected by INRA Rennes (France)
G4: XBJ92.10–27–1-1-1-1-1	Cross performed in Beja (Tunisia) in 1992 between faba bean breeding line selected for resistance to *O. crenata* by ICARDA and faba bean small seeds selected by INRAT
G5: XBJ92–10–46-1-3-1-2-1-1-1-6-A	Cross performed in Beja (Tunisia) in 1992 between faba bean breeding line selected for resistance to *O. crenata* by ICARDA and faba bean small seeds selected by INRAT
G6: XBJ90.04–6–2-1-1-4-C	Cross performed in Beja (Tunisia) in 1990 faba bean breeding line selected for resistance to *O. crenata* by ICARDA and faba bean small seeds local population
G7: XBJ90.04–2–3-1-1-1-2 A	Cross performed in Beja (Tunisia) in 1990 faba bean breeding line selected for resistance to *O. crenata* by ICARDA and faba bean small seeds local population
G8: Najeh	Small seeded variety released in 2009/partial resistant variety to *O. foetida* and *O. crenata*
G9: Badï	Small seeded variety released in 2004/susceptible to *O. foetida* and *O. crenata*

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