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Characterisation of resistance to branched broomrape, *Phelipanche ramosa*, in winter oilseed rape

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ABSTRACT

Over the past decade, *Phelipanche ramosa*, a weedy parasitic plant (broomrape), has been increasingly infesting winter oilseed rape (WOSR) fields in France. Elite WOSR lines have shown different responses in *P. ramosa* infested fields, suggesting that genetic variability might be available for breeding programmes targeting broomrape resistance. Ten WOSR genotypes selected for their contrasting response in field experiments were analysed using mini-rhizotron and greenhouse co-culture experiments to determine the components of resistance to broomrape. Partial resistance was revealed at three developmental stages of the parasitic plant. First, at the germination stage, parasite attachment to the roots of some WOSR lines was limited and associated with a low rate of parasite seed germination. This mechanism of parasite avoidance could nevertheless be suppressed under high infestation in mini-rhizotron and greenhouse conditions. Second, at the root attachment stage, limited parasite attachment was observed in mini-rhizotron conditions under low and high infestation, and in greenhouse conditions. Third, after successful parasite attachment, some WOSR genotypes retarded and even disturbed the growth of tubercles, minimising and delaying parasite emergence from the soil. Although the exact mechanisms limiting parasite attachment and tubercle development require further investigation, our findings suggest that, by cumulating various resistance traits in new genotypes to enhance effectiveness and potential durability of resistance, breeding could be a promising control strategy in WOSR.

1. Introduction

Broomrape species (*Orobanche* spp. and *Phelipanche* spp.) are obligate root parasitic plants devoid of chlorophyll. Some are harmful parasitic weeds in important crops, including *Orobanche cumana* Wallr. on sunflower, *Orobanche crenata* Forsk. and *Orobanche foetida* Poir. on legumes, and *Phelipanche ramosa* L. Pomel, (syn. *Orobanche ramosa* L. Pomel) and *Phelipanche aegyptiaca* Pers. on tomato (Parker, 2009). *P. ramosa* is by far the most widespread species due to its extremely wide range of host plants from cultivated species to weeds. Winter oilseed rape (WOSR, *Brassica napus* L.) is the primary European oilseed crop, and production areas have increased steadily since 2005, especially in France, Germany and in Eastern European countries (http://faostat.fao.org/). In addition to known virulent WOSR pathogens such as insects and fungi, branched broomrape (*P. ramosa*) has become a major phytosanitary problem in WOSR fields in western France over the past decade, causing heavy seed yield losses in highly infested areas (Gibot-Leclerc et al., 2001). High infestations in oilseed rape fields have previously been reported in Spain (Sobrino-Vesperinas, 1982) and led to abort oilseed rape culture in those regions. Recently, seed companies recorded serious cases of *P. ramosa* attacks in oilseed rape fields in other European countries (Bulgaria and Greece) due to the concurrent increase in oilseed rape crops and the absence of efficient control methods in these countries (Shindrova and Kostov, 2009; Tsialtas and Eleftherohorinos, 2011).

The weedy life cycle of broomrapes is well described in regard to its major host plants (Joel et al., 2007). Seed germination is induced by molecules exuded in the rhizosphere by host roots. Most germination stimulants identified so far belong to the strigolactone

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family (Yoneyama et al., 2010). Nevertheless, seeds of some *Orobanche* species such as *O. foetida* do not germinate in response to the synthetic strigolactone GR24 (Fernández-Aparicio et al., 2008), suggesting that compounds other than strigolactones are involved in broomrape seed germination. Some recent studies argue in favour of the involvement of dehydrocostus lactone, polyphenols and isothiocyanates in the germination of *O. cumana* (Joel et al., 2011), *O. foetida* (Evidente et al., 2010) and *P. ramosa* (Auger et al., 2012), respectively. Whatever the germination stimulant, germination leads to the emergence of a radicle that attaches to the host root surface. The parasitic phase starts with the penetration of the parasite into the host root through a differentiating haustorium, which connects to the host vascular tissues and serves as an attaching organ and as a bridge for water and nutrient transfer from the host. The parasite develops a tubercle, which gives rise to a subterranean shoot and then, after emergence from the soil, a branched flowering spike.

Over the past decade, several studies have sought to evaluate the resistance of host plants to broomrape species in the field (Rubiales and Fernández-Aparicio, 2011). Resistance to broomrapes is rare and most often only partial (Rubiales, 2003). Early resistance is expressed during the pre-attachment phase and includes a low induction of seed germination by host roots (Fernández-Aparicio et al., 2012), the exudation of parasitic seed germination inhibitors (Mabrouk et al., 2007) and/or phytoalexins (Serghini et al., 2001), and cell wall reinforcement that blocks parasite penetration and connection to the host vascular system (Labrousse et al., 2001; Echevarría-Zomeño et al., 2006). Late resistance is expressed after the vascular connection between the host and the parasite and mainly leads to parasite necrosis due to host vessel occlusion by mucilage, unidentified host-degraded products or production of toxic compounds by the host, as observed in sunflower (Labrousse et al., 2001; Letousey et al., 2007; de Zélicourt et al., 2007) and legumes (Pérez-de-Luque et al., 2006; Fernández-Aparicio et al., 2009, 2012).

Several strategies have been employed to control broomrapes, but none has enjoyed unequivocal success (Pérez-de-Luque et al., 2009; Rubiales and Fernández-Aparicio, 2011). Breeding for resistance is the most economic, feasible and environmentally friendly method of control. Nevertheless, to avoid the emergence of new pathogenic races that can overcome the prevailing resistance genes, as observed in the sunflower – *O. cumana* pathosystem (Molinero-Ruiz et al., 2009), the production of new resistant lines cumulating several resistance traits is strongly recommended. Also, better knowledge of the mechanisms involved in resistance is needed.

Damage of WOSR by *P. ramosa* is a recent economic concern in comparison to other plant–parasitic weed interactions such as legumes – *O. crenata* and sunflower – *O. cumana*. Consequently, there is little information on broomrape resistance in oilseed rape. In Spain, Sobrino-Vesperinas (1985) searched for sources of resistance in 33 rapeseed cultivars and breeding lines but did not detect any complete resistance. Similarly, 15 varieties of oilseed rape have been screened in greenhouse conditions under artificial infestation (Zehhar et al., 2003) and all were susceptible, although there was significant variability in the screened material. Another study explored the susceptibility of two different oilseed rape cultivars (summer and winter oilseed rape) to German populations of *P. ramosa* (Buschmann et al., 2005). Similarly, studies based on genetic analysis and cross infections have showed the existence of three French populations of *P. ramosa* (pathovars) showing inter-population genetic diversity with an adaptation with regards to the host plant (WOSR, tobacco and hemp) (Benharrat et al., 2005; Brault et al., 2007). Depending on the *P. ramosa* population, four oilseed rape varieties showed divergent susceptibility to infection (Benharrat et al., 2005). Oilseed rape breeding programs started in the 1950s to promote better adaptation to pedoclimatic conditions and led to substantial success in cold resistance. During the 1970s and 1980s, breeders offered double low (00) varieties with reduced levels of erucic acid and glucosinolates, making rapeseed oil suitable for human consumption and rapeseed meal an excellent protein source for animal feed. Regarding resistance to pathogens, breeding has been mainly conducted to control phoma stem canker caused by *Leptosphaeria maculans* Desm. (Ces & de Not) (Delourme et al., 2006).

Due to the alarming expansion of *P. ramosa* in its most recent host crop, oilseed rape, in France, one of the tasks of the French Technical Centre for Oilseed Crops (CETIOM) consists in evaluating the response of elite WOSR lines towards *P. ramosa* in strongly infested areas in central western France. In these field experimentations, although all lines bore emerged broomrapes at the harvest date, there was contrasting response among the tested lines when overall development was evaluated (CETIOM, Pers. Comm.). This variability may be due to resistance and/or tolerance traits and is encouraging for breeding for resistance in WOSR. Therefore, using artificial infestation in mini-rhizotron (Petri dishes) and greenhouse (pots) conditions, the present study further characterises the resistance of elite WOSR lines that showed contrasting response in broomrape-infested fields.

2. Materials and methods

2.1. Plant materials

Seeds from *P. ramosa* pathovar adapted to oilseed rape (Benharrat et al., 2005) were collected from flowering spikes in WOSR fields in central western France (Saint-Martin-de-Freigneau station, 2007) and stored in the dark at 25 °C until use. Seeds of elite WOSR varieties were provided by seed companies, the French National Institute for Agricultural Research (INRA) and CETIOM. Among the 10 WOSR lines used in the present study (Table 1), 8 were selected for their contrasting response to *P. ramosa* in infested fields in central western France over the last five crop seasons (CETIOM, unpublished data), and 2 other lines were used as controls, because they showed contrasted interaction with *P. ramosa* in previous studies: Darmor is considered as a “resistant” variety and Yudal a “susceptible” variety (Véronési et al., 2006, 2009).

2.2. Mini-rhizotron experiments

WOSR and *P. ramosa* seeds were surface-sterilised in 12% sodium hypochlorite for 5 min and then rinsed with sterile distilled

Table 1
Field response of the Elite WOSR lines used in the present study.

Genotype	Seed producer	Response towards *P. ramosa*[a]
Adriana	Advanta	++
Alesi	KWS maize (France)	+
Aviso	Advanta	+
Campo	Dekalb	–
Cooper	Advanta	++
Darmor	INRA	nd
Expert	Momont	nd
Grizzly	RAGT seeds	–
Shakira	Maïsadour seeds	–
Yudal	INRA	nd

[a] Response scores according to the impact of infestation on overall development and fruit yield during the last five crop seasons: –, no significant impact; +, moderate impact; ++, strong impact; n.d, not determined.

water. WOSR seeds were placed in 19 cm Petri dishes between two layers of glass fibre for 7 days at 23 °C, with 300 mmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and a 16 h photoperiod in a growth chamber (Froid et Mesures, Beaucouzé, France). Plantlets were transferred into 25 × 25 cm Petri dishes (one plantlet per Petri dish) on glass fibre paper and mineral wool, fertilised with 50% Coïc medium (Coïc and Lesaint, 1975), and cultivated for four weeks in growth chamber before inoculating them with broomrape. Sterilised broomrape seeds were conditioned on two layers of glass fibre in Petri dishes following a seven-day period in the dark at 23 °C. Inoculation consisted in placing conditioned broomrape seeds (20 mg FW per Petri dish, density of about 15 seeds cm^{-2}) on the glass fibre filter paper close to host roots. Rate of broomrape seed germination in the vicinity of WOSR roots was assayed 12, 19, 26 and 33 days post infection (dpi). To do so, the same 400 *P. ramosa* seeds, close to host roots (<5 mm) and distributed in four identified squares, were observed per Petri dish at every date using a stereoscopic microscope (Olympus Optical, Tokyo, Japan) at 30× magnification to determine the germination rate, expressed as the percentage of seeds with an emerged radicle (at least 1.5 mm length).

In another experiment, inoculation was done with germinated seeds. Germination was induced by adding GR24 ($3 \cdot 10^{-8}$ M, optimal concentration), a synthetic strigolactone (Johnson et al., 1976). After 30 h of GR24 treatment, seeds were washed twice in distilled water to remove excess GR24, and then placed close to WOSR roots as with conditioned seeds.

The total number of broomrape attachments per host plant was monitored weekly for 75 dpi and every tubercle was classified according its developmental stage as previously defined by Labrousse et al. (2001): stage 1, germinated broomrape seed attachment to the host root; stage 2, tubercle formation; stage 3, tubercle bearing numerous adventitious roots; stage 4, tubercle bearing a growing shoot; stage 5, tubercle bearing a flowering shoot. At 75 dpi, WOSR roots were harvested and dried in an oven at 80 °C for 48 h after discarding broomrape tubercles. The total number of *P. ramosa* tubercles is expressed as the number of tubercles per g of WOSR root dry weight (number/g root DW). Due to the strong development of rapeseed roots covering the total surface of filters, expression of infection rates in number/g root DW was more suitable than in number/cm root as done for other host plants (Fernández-Aparicio et al., 2009). In any case, root systems did not show visually significant variation in root length. The median time from inoculation, at which 50% of the maximum number of attachments was reached (T_{50}), was calculated as previously described (Abbes et al., 2010).

The two mini-rhizotron experiments with conditioned seeds or GR24 germinated seeds were conducted with 10 and 4 rapeseed genotypes, respectively, and were repeated twice in independent manner using ten plants per genotype and per repetition.

2.3. Pot experiments

Based on the results from mini-rhizotron experiments, five WOSR genotypes (Darmor, Campo, Shakira, Yudal and Grizzly) were further characterised in greenhouse pot experiments. Broomrape seeds (5 mg) were mixed with a peat-sand-clay mixture (1:1:1 vol) in 0.4 L 'Deep Jiffy Peat Pots' which were placed in 1.3 L plastic pots containing uninfected soil. Broomrape seeds were conditioned by protecting watered pots from light for a week at 23 °C. Three WOSR seeds were then sown directly into each pot. Two weeks after WOSR emergence, seedlings were thinned to one per pot. Plants were grown at 23 ± 5 °C with 300 μmol m^{-2} s^{-1} PAR and a 16 h photoperiod, and fertilised with 3‰ Lycoplant blue (Plantin, Courthéson, France) once or twice a week.

Fourteen weeks after sowing, WOSR plants were removed from their pots, broomrape tubercles were removed, and roots were carefully washed in water, harvested and dried in an oven at 80 °C for 48 h. The number of tubercles is expressed as the total number of tubercles per g of root dry weight (number/g root DW). Over the course of the experiment, the kinetic of parasite emergence was assessed daily. The date of first broomrape emergence is expressed as the average number of days after sowing when the first broomrape emerged from the soil in each pot. Pot experiments were repeated twice in independent manner using ten plants per genotype and per repetition.

2.4. Statistical analysis

The experiments were arranged randomised with ten replicates per WORS genotype. Statistical analysis was conducted to the combined data of replicated experiments (homogenous variances). Analysis of variance (ANOVA) was conducted on the number of attached or emerged broomrapes, using R software (http://wwwR-project.org) and the Rcmdr package (http://CRAN.R-project.org/package=Rcmdr), and with genotype and GR24 treatment as factors. Significant differences between the means were determined by Tukey's HSD test at $P < 0.05$. Before performing analyses of variance, the normality and homogeneity of variances were confirmed.

3. Results

3.1. Mini-rhizotron experiments

Using mini-rhizotron experiments, we accurately characterised the *B. napus*–*P. ramosa* interaction, from induction of parasitic seed germination to the advanced stage of parasite development (tubercles with flowering shoots). Fig. 1 shows that parasite seed germination (33 dpi) and tubercle production (75 dpi) varied among the 10 tested WOSR genotypes. Weekly evaluation of the germination rate (during the first 33 days) revealed that maximum germination was reached as of 12 dpi for all the studied lines (data not shown). WOSR genotypes differed in their capacity to induce germination of conditioned *P. ramosa* seeds (Fig. 1). For example, the germination rate ranged from 15.0 ± 5.0% for Darmor to almost 83.0 ± 2.8% for Cooper. Similarly, the mean tubercle production varied among the genotypes and ranged from 0.5 ± 0.3 for Darmor to 10.2 ± 1.5 tubercles/g root DW for Grizzly. Examined together, these results suggest that the number of parasite tubercles does not depend only on the capacity of host roots to induce parasite seed germination. For instance, Darmor, which is the least-inducing genotype, bore the lowest number of broomrape tubercles. Conversely, Grizzly, which was one of the genotypes that strongly induced germination of parasite seeds (79 ± 3.5%), was severely infected (10.2 ± 1.5 tubercles/g root DW) by *P. ramosa*. However, although Expert and Adriana stimulated broomrape seed germination as much as Grizzly did, they were significantly less infested. Thus, although the capacity of the WOSR genotype to induce the germination of broomrape seeds is an essential element, some additional elements control the successfulness of broomrape attachment in oilseed rape.

To evaluate the capacity of host roots to promote the attachment of seedlings independently of their capacity to induce parasite seed germination, root infection was tested with GR24-germinated seeds instead of conditioned seeds. Experiments were performed on four WOSR genotypes showing contrasting capacity to induce germination of conditioned seeds: Darmor, Campo, Shakira and Grizzly (Fig. 2). The rate of seed germination following GR24 treatment was around 85%. For Grizzly, the number of tubercles

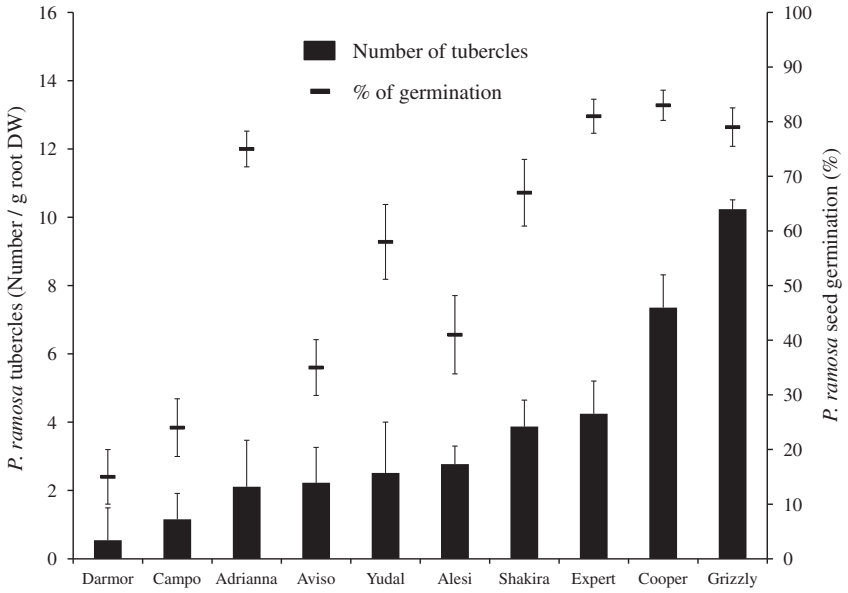

Fig. 1. Germination rates of *P. ramosa* seeds (33 dpi) and the number of parasite tubercles per gram of oilseed rape root dry weight (75 dpi) in mini-rhizotron experiments in ten oilseed rape genotypes. Data are means and lines indicate 95% confidence intervals ($n = 20$).

arising from conditioned and pre-germinated seeds was not statistically different 75 dpi (10.2 ± 1.5 *vs* 9.8 ± 1.2 tubercles/g root DW). This result was coherent since this genotype stimulated germination of conditioned *P. ramosa* seeds at a rate similar to the GR24-treated seeds. Consequently, the GR24 treatment did not modify parasitic pressure. In contrast, for Darmor, Campo and Shakira genotypes, the number of tubercles differed greatly between the two conditions (0.7 ± 0.5 *vs* 5.6 ± 2.3, 1.2 ± 0.5 *vs* 6.1 ± 2.5 and 4.2 ± 1.3 *vs* 12.0 ± 3.2 tubercles/g root DW, respectively; Fig. 2), demonstrating that the number of broomrape attachments significantly increased with artificial induction of parasite seed germination before host root infection. However, significant differences in the degree of infection were observed among the tested genotypes: there were twice as many tubercles on Shakira and Grizzly root systems than on Darmor and Campo roots, both of which bore a similar number of tubercles (Fig. 2).

The kinetics of tubercle development according to host genotype was studied to identify possible post-attachment resistance mechanisms. This was carried out by recording weekly changes in the number of tubercles and their development for 75 dpi (Table 2). First, with respect to the total number of tubercles per plant, Cooper and Grizzly bore significantly higher numbers of tubercles than the other genotypes from 12 dpi on and throughout the experiment. Conversely, Darmor and Campo, exhibited significantly

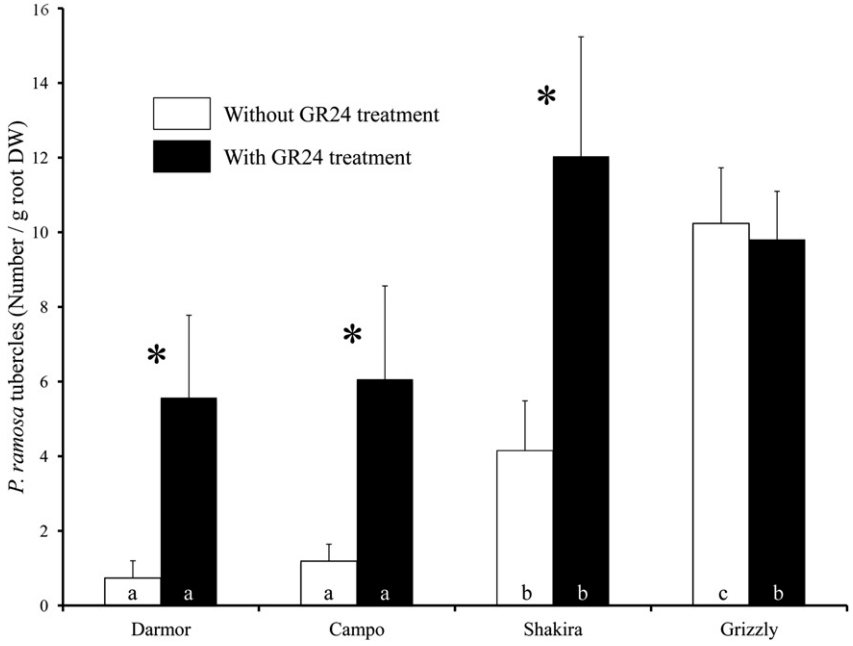

Fig. 2. Number of *P. ramosa* tubercles on four oilseed rape genotypes per gram of root dry weight (75 dpi) in mini-rhizotron experiments. Germination of parasite seeds without (white bars) or with (black bars) a GR24 treatment. Data are means and lines indicate standard error. For a given genotype, asterisks indicate that values are significantly different between the two conditions (Tukey's HSD test, $P < 0.05$). For a given condition, genotypes with the same letter are not significantly different (Tukey's HSD test, $P < 0.05$).

Table 2
Kinetics of development of *P. ramosa* tubercles on winter oilseed rape genotypes in hydroponic experiments.

Dpi	Stage*	Grizzly	Cooper	Expert	Shakira	Alesi	Yudal	Aviso	Adriana	Campo	Darmor
12	1	15.6 ± 5.8	11.8 ± 8.6	4.3 ± 2.4	3.3 ± 3.1	3.0 ± 3.9	1.7 ± 1.2	4.5 ± 3.9	3.0 ± 1.8	0.5 ± 0.5	0
	2	3.5 ± 3.9	1.0 ± 1.7	1.3 ± 1.4	0	0.7 ± 1.6	0.5 ± 1.2	0.7 ± 1.0	2.3 ± 1.9	0	0
	3	0.1 ± 0.4	0	0.1 ± 0.4	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		19.3 ± 8.4^{c}	12.8 ± 8.0^{c}	5.6 ± 3.3^{b}	3.3 ± 3.1^{ab}	3.7 ± 5.3^{ab}	2.2 ± 2.1^{ab}	5.2 ± 4.0^{b}	5.3 ± 2.4^{b}	0.5 ± 0.5^{a}	0^{a}
19	1	5.9 ± 3.4	5.0 ± 2.5	1.5 ± 0.9	4.7 ± 2.3	2.3 ± 3.3	1.0 ± 1.3	1.3 ± 1.4	2.8 ± 1.5	0.7 ± 0.5	0
	2	24.4 ± 4.4	17.2 ± 12.6	8.9 ± 5.2	6.5 ± 3.7	4.5 ± 5.5	3.2 ± 3.1	4.3 ± 3.9	3.3 ± 3.1	0.5 ± 1.2	0.3 ± 0.5
	3	13 ± 8.1	4.2 ± 4.4	8.5 ± 5.1	1.3 ± 1.8	2.3 ± 3.0	0.2 ± 0.4	1.5 ± 1.2	3.0 ± 2.1	0	0
	4	0	0	0.3 ± 0.5	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		43.3 ± 10.3^{d}	26.3 ± 15.9^{c}	19.1 ± 10.1^{bc}	12.5 ± 5.5^{b}	9.2 ± 11.5^{ab}	4.3 ± 2.6^{ab}	7.2 ± 4.2^{b}	9.2 ± 5.7^{b}	1.2 ± 1.5^{a}	0.3 ± 0.5^{a}
26	1	4.0 ± 2.4	2.7 ± 2.9	1.0 ± 0.9	3.3 ± 2.3	2.2 ± 2.7	0.2 ± 0.4	1.2 ± 1.2	2.0 ± 1.3	0.7 ± 0.5	0.1 ± 0.4
	2	6.8 ± 2.3	16.5 ± 5.3	2.1 ± 1.6	5.0 ± 3.4	2.3 ± 3.0	4.0 ± 2.2	3.5 ± 5.5	1.5 ± 2.1	1.0 ± 1.7	0.1 ± 0.4
	3	37.0 ± 12.6	22.0 ± 15.1	13.5 ± 7.8	9.3 ± 3	6.7 ± 7.2	6.7 ± 5.2	4.7 ± 4.3	6.5 ± 5.1	0.8 ± 1.6	0.4 ± 0.8
	4	0.4 ± 1.1	0	4.6 ± 6.6	0	0.5 ± 0.5	1.7 ± 3.1	0.3 ± 0.5	0.2 ± 0.4	0	0
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		48.1 ± 10.4^{c}	41.2 ± 17.6^{c}	21.3 ± 11.3^{b}	17.7 ± 6.2^{b}	11.7 ± 12.4^{b}	12.5 ± 6.7^{b}	9.7 ± 6.3^{b}	10.2 ± 6.0^{b}	2.5 ± 3.1^{a}	0.7 ± 1.0^{a}
33	1	3.6 ± 2.4	2.5 ± 2.6	1.0 ± 0.9	3.2 ± 2.6	1.7 ± 2.1	0.2 ± 0.4	0.8 ± 1.0	1.7 ± 1.0	0.7 ± 0.5	0.4 ± 0.8
	2	4.4 ± 1.6	1.7 ± 1.6	0.9 ± 1.0	3.7 ± 2.0	1.3 ± 1.2	1.3 ± 1.2	2 ± 2.5	0.8 ± 0.8	0.7 ± 1.2	0
	3	38.9 ± 9.5	32.2 ± 15.2	9.1 ± 5.6	11.3 ± 3.6	9.8 ± 10.3	8.5 ± 5.9	7.3 ± 4.7	8.2 ± 5.4	1.8 ± 2.2	0.7 ± 1.1
	4	2.9 ± 2.6	9.2 ± 6.5	12.3 ± 12.5	0.7 ± 0.8	1.3 ± 1.2	3.7 ± 3.8	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.8	0.4 ± 0.8
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		49.8 ± 9.2^{d}	45.5 ± 17.1^{d}	23.3 ± 11.4^{c}	18.8 ± 5.5^{bc}	14.2 ± 13.1^{b}	13.7 ± 6.2^{bc}	10.5 ± 6.9^{b}	11.0 ± 5.6^{b}	3.5 ± 3.4^{ab}	1.6 ± 1.5^{a}
40	1	3.4 ± 2.6	2.3 ± 3.0	1.0 ± 0.9	3.0 ± 2.4	1.8 ± 2.4	0.2 ± 0.4	0.8 ± 1.0	1.5 ± 1.2	0.7 ± 0.5	0.4 ± 0.8
	2	2.6 ± 1.7	0.2 ± 0.4	0.3 ± 0.5	2.0 ± 2.4	1.7 ± 1.6	0.3 ± 0.8	1.5 ± 2.7	0.5 ± 0.5	0.5 ± 0.8	0
	3	38.6 ± 9.8	32.2 ± 15.1	9.5 ± 5.7	12.3 ± 3.5	5.0 ± 4.4	3.2 ± 2.4	3.7 ± 2.3	7.8 ± 5.6	2.5 ± 2.1	1.0 ± 1.5
	4	5.3 ± 3.2	12.2 ± 6.1	12.9 ± 12.7	1.7 ± 1.4	6.7 ± 8.4	10.3 ± 6.7	5.7 ± 4.7	1.5 ± 1.4	0.8 ± 1.3	0.6 ± 1.1
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		49.9 ± 9.5^{d}	46.8 ± 16.6^{d}	23.6 ± 11.4^{c}	19.0 ± 5.5^{bc}	15.2 ± 14.0^{b}	14.0 ± 6.1^{bc}	11.7 ± 6.2^{b}	11.3 ± 5.3^{b}	4.5 ± 4.0^{ab}	2.0 ± 1.6^{a}
47	1	3.1 ± 2.4	2.3 ± 3.0	1.0 ± 0.9	2.8 ± 2.8	1.7 ± 2.1	0.2 ± 0.4	0.8 ± 1.0	1.5 ± 1.2	0.7 ± 0.5	0.4 ± 0.8
	2	2.9 ± 1.5	0.2 ± 0.4	0.3 ± 0.5	2.0 ± 2.4	1.5 ± 1.4	0.3 ± 0.8	1.3 ± 2.8	0.3 ± 0.5	0.5 ± 0.8	0
	3	20.3 ± 10.7	6.0 ± 9.1	5.4 ± 5.1	13.0 ± 2.8	4.3 ± 3.5	1.3 ± 2.4	4.2 ± 2.2	9.0 ± 5.3	1.8 ± 1.8	1.0 ± 1.5
	4	25.1 ± 10.2	38.3 ± 12.8	17.9 ± 12.3	2.0 ± 1.3	7.7 ± 9.6	12.5 ± 6.7	5.8 ± 4.8	1.2 ± 0.8	2.3 ± 2.6	0.6 ± 1.1
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		51.4 ± 11.2^{d}	46.8 ± 16.4^{d}	24.5 ± 11.3^{c}	19.8 ± 5.1^{bc}	15.2 ± 14.0^{b}	14.3 ± 6.8^{bc}	12.2 ± 5.9^{b}	12.0 ± 5.6^{b}	5.3 ± 4.3^{ab}	2.0 ± 1.6^{a}
54	1	2.5 ± 1.7	2.2 ± 3.0	1.0 ± 0.9	2.8 ± 2.8	1.7 ± 2.1	0.2 ± 0.4	0.8 ± 1.0	1.5 ± 1.2	0.5 ± 0.5	0.4 ± 0.8
	2	2.9 ± 1.5	0.2 ± 0.4	0.3 ± 0.5	2.0 ± 2.4	1.5 ± 1.0	0.3 ± 0.8	1.3 ± 2.8	0.3 ± 0.5	0.3 ± 0.5	0
	3	17.3 ± 9.2	2.5 ± 1.8	5.1 ± 5.1	12.5 ± 3.1	4.2 ± 3.3	1.0 ± 1.7	3.7 ± 2.4	7.8 ± 4.6	2.5 ± 1.9	0.7 ± 1.1
	4	29 ± 9.7	42 ± 17.5	18.1 ± 12.2	2.5 ± 1.6	8.5 ± 10.3	13.0 ± 6.3	6.3 ± 5.0	2.3 ± 1.4	2.3 ± 2.6	0.9 ± 1.2
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		51.6 ± 11.0^{d}	46.8 ± 16.4^{d}	24.5 ± 11.3^{c}	19.8 ± 5.1^{bc}	15.8 ± 13.8^{b}	14.5 ± 6.9^{bc}	12.2 ± 5.9^{b}	12.0 ± 5.6^{b}	5.7 ± 4.4^{ab}	2.0 ± 1.6^{a}
61	1	2.4 ± 1.7	1.5 ± 2.0	1.0 ± 0.9	2.8 ± 2.8	1.7 ± 2.1	0.2 ± 0.4	0.8 ± 1.0	1.5 ± 1.2	0.3 ± 0.5	0.4 ± 0.8
	2	2.9 ± 1.5	0.2 ± 0.4	0.3 ± 0.5	2.0 ± 2.4	1.5 ± 1.0	0.3 ± 0.8	1.3 ± 2.8	0.5 ± 0.5	0.2 ± 0.4	0
	3	13.3 ± 8.6	3.7 ± 1.9	4.6 ± 5.0	12.5 ± 3.1	3.3 ± 3.3	0.7 ± 1.6	3.7 ± 2.5	5.2 ± 3.4	3.2 ± 1.8	1.0 ± 1.8
	4	33.3 ± 10.4	42.2 ± 17.5	18.6 ± 12.3	2.5 ± 1.6	9.3 ± 11.7	13.3 ± 7	6.7 ± 5.1	5.0 ± 2.7	3.3 ± 3.2	1.3 ± 2
	5	0	0	0	0	0	0	0	0	0	0
No. tubercles/plant**		51.8 ± 11.0^{c}	47.5 ± 16.7^{c}	24.5 ± 11.3^{b}	19.8 ± 5.1^{b}	15.8 ± 13.8^{b}	14.5 ± 6.9^{b}	12.5 ± 5.9^{b}	12.2 ± 5.5^{b}	7.0 ± 4.0^{a}	2.7 ± 2.1^{a}
68	1	2.4 ± 1.7	1.5 ± 2.0	1.0 ± 0.9	2.8 ± 2.8	1.7 ± 2.1	0.2 ± 0.4	0.8 ± 1.0	1.5 ± 1.2	0.3 ± 0.5	0.4 ± 0.8
	2	2.9 ± 1.5	0.2 ± 0.4	0.3 ± 0.5	2.0 ± 2.4	1.5 ± 1.0	0.3 ± 0.8	1.3 ± 2.8	0.3 ± 0.5	0.2 ± 0.4	0
	3	11.6 ± 6.1	3.5 ± 2.1	3.5 ± 4.8	8.3 ± 3.0	3.5 ± 3.6	0.7 ± 1.6	3.7 ± 2.7	4.3 ± 2.4	3.2 ± 1.8	1.1 ± 1.3
	4	35.4 ± 10.8	42.3 ± 17.5	18.1 ± 11.5	7.7 ± 1.9	9.3 ± 11.7	11.7 ± 6.5	6.7 ± 5.5	6.0 ± 3.8	3.3 ± 3.2	1.1 ± 1.2
	5	0	0	1.6 ± 3.1	0	0	1.7 ± 1.4	0.3 ± 0.5	0	0	0.4 ± 0.8
No. tubercles/plant**		52.3 ± 11.1^{d}	47.5 ± 16.7^{d}	24.5 ± 11.3^{c}	20.8 ± 5.1^{bc}	16 ± 13.9^{b}	14.5 ± 6.9^{bc}	12.8 ± 5.6^{b}	12.2 ± 5.5^{b}	7.0 ± 4.0^{ab}	3.1 ± 2.1^{a}
75	1	2.4 ± 1.7	1.5 ± 2.0	1.0 ± 0.9	2.8 ± 2.8	1.7 ± 2.1	0.2 ± 0.4	0.8 ± 1.0	1.5 ± 1.2	0.3 ± 0.5	0.4 ± 0.8
	2	2.9 ± 1.5	0.2 ± 0.4	0.3 ± 0.5	2.0 ± 2.4	1.5 ± 1.0	0.3 ± 0.8	1.3 ± 2.8	0.3 ± 0.5	0.2 ± 0.4	0
	3	11.4 ± 6.5	2.3 ± 2.7	3.5 ± 4.8	7.2 ± 3.1	2.8 ± 3.7	0.7 ± 1.6	3.2 ± 3.1	3.5 ± 2.3	3.3 ± 1.6	1.0 ± 1.4
	4	35.6 ± 11.2	42.0 ± 14.7	15 ± 9.4	8.8 ± 1.2	9.7 ± 12	9.5 ± 5.9	7.2 ± 5.4	6.8 ± 5.0	3.3 ± 3.2	1.1 ± 0.9
	5	0	1.5 ± 1.6	4.8 ± 4.8	0	0.3 ± 0.5	3.8 ± 3.1	0.5 ± 0.8	0	0	0.6 ± 1.1
No. tubercles/plant**		52.3 ± 11.1^{d}	47.5 ± 16.7^{d}	24.5 ± 11.3^{c}	20.8 ± 5.1^{bc}	16.0 ± 13.9^{b}	14.5 ± 6.9^{bc}	13.0 ± 5.5^{b}	12.2 ± 5.5^{b}	7.2 ± 3.7^{ab}	3.1 ± 2.1^{a}
Transition 2–3 in days		26	26	26	26	26	26	26	26	n.d	n.d
Transition 3–4 in days		47	47	33	75	40	40	40	68	n.d	n.d
T_{50} in days		13.4 ± 0.2	16.0 ± 0.5	15.1 ± 0.4	17.1 ± 0.4	17.9 ± 0.4	21.3 ± 0.6	16.6 ± 0.8	13.1 ± 0.4	n.d	n.d

*Developmental stage scored according to the Labrousse et al. (2001) scale, where 1 = germinated broomrape seed attachment to host root; 2 = tubercle formation; 3 = tubercle bearing numerous adventitious roots; 4 = tubercle bearing a growing shoot; 5 = tubercle bearing a flowering shoot.
**Values with the same letter (in a single row) are not significantly different (Tukey's HSD test, $P < 0.05$).
T_{50}, median time from inoculation to 50% of the maximum number of attachments. n.d., not determined.

fewer tubercles than the other genotypes. Regardless of these divergences, the number of days required for 50% of the tubercles to be produced (T_{50}) was similar for all four genotypes (around 16 dpi), indicating that the global kinetics of tubercle production were similar among these genotypes. However, genotypes varied in their kinetics of tubercle development. Except for Darmor and Campo, for which the number of tubercles was too low to analyse, the date of developmental transition corresponding to a greater number of stage $n + 1$ tubercles compared to stage n tubercles (transition 2–3 and 3–4) could be determined from weekly

records. Regardless of host genotype, the developmental transition 2–3 occurred at 26 dpi, indicating that the early development of tubercles proceeded at a similar rate without any limiting resistance mechanism. In contrast, the kinetics of later development of tubercles depended on host genotype. The date of developmental transition 3–4 ranged from 33 dpi for Expert to 75 dpi for Shakira. There was no correlation between the late kinetics of tubercle development and the number of tubercles. Yudal and Adriana, both of which bore a low number of tubercles (14.5 ± 6.9 and 12.2 ± 5.5, respectively), differed significantly in their developmental transition 3–4, 40 and 68 dpi, respectively. In decreasing order of tubercle development, Expert was the genotype that allowed the fastest development of established tubercles (transition 3–4, 33 dpi), followed by Alesi, Aviso and Yudal (transition 3–4, 40 dpi) with a similar severity of infection (on average, 14.5 tubercles/plant); genotypes Grizzly and Cooper facilitated rapid attachment of *P. ramosa*: the first stage 1 attachments appeared early (as of 12 dpi), but transition 3–4 occurred later (47 dpi); Adriana showed delayed developmental transition 3–4 (68 dpi) as did Shakira, which even blocked tubercle development as of stage 3. Moreover, parasite development was drastically slowed in the Shakira root system, with developmental transition 3–4 occurring at only 75 dpi. In this system, *P. ramosa* plants frequently showed a large tubercle phenotype. Taken together, our results demonstrate that WOSR genotypes may act differentially on tubercle development following parasite attachment, suggesting the involvement of uncharacterised late resistance mechanisms during the post-attachment stages of parasitism.

3.2. Pot experiments

Five reference genotypes showing contrasting response in mini-rhizotron experiments (Darmor, Campo, Shakira, Grizzly and Yudal) were further studied in greenhouse pot experiments to compare their responses in other growth conditions (Table 3). The mean number of *P. ramosa* tubercles per g of root DW was not significantly different 105 days after sowing in genotypes Campo (4.8 ± 1.1), Shakira (5.1 ± 1.7), Grizzly (5.9 ± 1.7) and Yudal (6.2 ± 2.2), while Darmor bore significantly fewer tubercles (1.9 ± 0.8). The average date at which the first shoots emerged from the soil was 89, 90, 93 and 96 days after sowing for the genotypes Yudal, Darmor, Campo, and Grizzly, respectively. In Shakira, shoots emerged significantly later: 102 days after sowing. Only broomrapes attached to Shakira roots exhibited a specific phenotype consisting of large tubercles with thin shoots.

4. Discussion

While sources of resistance to broomrape species have been already reported in several cultivated species (Pérez-de-Luque et al., 2009; Rubiales and Fernández-Aparicio, 2011), little is known about *B. napus* germplasm regarding resistance to *P. ramosa*. The *B. napus*–*P. ramosa* interaction can be considered as recent economic concern to other plant–parasitic plant interactions, and breeding for *P. ramosa* resistance was not developed in *B. napus* as intensely as in other crops that have been challenged for a long time with broomrape, e.g. legumes and sunflower. The *P. ramosa* problem in French WOSR fields is very recent and has become a real problem since the mid-1990s. We thus sought to identify sources of resistance to *P. ramosa* in elite WOSR lines and to elucidate potential mechanisms of resistance.

Although no complete resistance was detected in any of the tested genotypes, different responses to *P. ramosa* infection were observed by investigating the host–parasite interaction using mini-rhizotron and greenhouse experiments. These responses can be related to mechanisms of parasite avoidance and partial resistance, in contrast to sunflower where resistance genes (*Or1–5*) confer total resistance to *O. cumana* races (Vranceanu et al., 1980). First, mini-rhizotron experiments showed that the severity of infection is partly related to the rate of broomrape seed germination in the vicinity of host roots. All tested genotypes developed well in mini-rhizotron conditions and the rate of parasite seed germination can range from a very low level (e.g. genotype Darmor, $15.0 \pm 5.0\%$) to a high level (e.g. genotype Cooper, $83.0 \pm 2.8\%$). Differences in germination rates probably reflect significant variability in the production of germination stimulants in *B. napus*, as reported in several other crops challenged with broomrape, including sunflower (Labrousse et al., 2001) and legumes (Rubiales et al., 2003; Sillero et al., 2005; Fernández-Aparicio and Rubiales, 2012), and sorghum challenged with the chlorophyllous parasitic plant *Striga hermonthica* (Del.) Benth (Haussman et al., 2001). Parasite avoidance due to low or moderate capacities to induce parasite germination plays a major role in resistance of genotypes Darmor, Campo and Shakira in mini-rhizotron conditions. In these genotypes, resistance was partially suppressed under artificially high infestation when WOSR roots were exposed to GR24 pre-germinated seeds. Except for Grizzly, this treatment dramatically increased the number of broomrape tubercles per plant. Shakira with a two-fold increase in broomrape attachment showed the highest level of susceptibility in this treatment. Similarly, parasite avoidance was artificially suppressed in the non-host *Arabidopsis thaliana* (L.) Heynh. when infested by GR24-stimulated *O. crenata* or *Orobanche minor* Sm. seeds (Westwood, 2000). Moreover, in our experiments, despite an about six-fold increase, establishment of broomrape tubercles on Darmor and Campo roots was shown to be significantly limited under a similar high parasitic pressure compared with tubercle establishment on Shakira and Grizzly roots. Limited tubercle establishment in high densities of germinated parasite seeds can be considered as a second resistance component. This resistance is also found in the genotypes Adrianna and Expert, in a lesser extent, which showed a high capacity to stimulate seed germination but bore a limited number of parasite tubercles as shown in Fig. 1. A barrier-type mechanism limiting parasite attachment has been reported in resistant chickpea accessions following a GR24-pre-germination treatment and was associated with an apparent “hypersensitive-like” reaction (Rubiales et al., 2003). Regarding the barriers limiting parasite attachment in WOSR under high parasitic pressure, no ‘hypersensitive’ reactions were observed. Although the set-up of physical and chemical barriers can be suggested as previously described (Pérez-de-Luque et al., 2009), further analyses need to be carried out to understand the underlying mechanisms.

A third component of WOSR resistance occurring later in tubercle development was identified. While tubercle development was not limited in parasites that had successfully established on the

Table 3
Response of winter oilseed rape genotypes to *P. ramosa* in pot experiments compared to hydroponic experiments.

Genotype	No. of *P. ramosa* tubercles/g root DW in pot experiments*/**	Date of first emergence in days after sowing in pot experiments**	*P. ramosa* germination rate/infection intensity in hydroponic experiments
Darmor	1.9 ± 0.8^{a}	90.4 ± 7.1^{a}	Low/low
Campo	4.8 ± 1.1^{b}	92.8 ± 5.6^{a}	Low/low
Shakira	5.1 ± 1.7^{b}	101.7 ± 6.2^{b}	Moderate/moderate
Grizzly	5.9 ± 1.7^{b}	96.3 ± 10.8^{ab}	High/high
Yudal	6.2 ± 2.2^{b}	88.9 ± 6.6^{a}	Moderate/moderate

*Data collected 105 days after sowing.
**Values with the same letter (in a given column) are not significantly different (Tukey's HSD test, $P < 0.05$).

highly susceptible genotypes Grizzly and Cooper (in which developmental transition 3–4 occurred as early as 47 dpi), tubercles found on Shakira roots showed delayed development, with developmental transition 3–4 occurring more than 20 days later (75 dpi). Moreover, the parasite growing on Shakira roots displayed a particular phenotype characterised by large tubercles with thin shoots. This kind of resistance has also been observed in some resistant legume accessions in which the development of *O. crenata* broomrape tubercles was stopped at an early stage or retarded (Sillero et al., 2005; Fernández-Aparicio et al., 2009). In our experiments, the enhanced radial growth of tubercles and the limited parasite shoot development on Shakira roots suggest deregulation of hormone levels and an alteration of parasite sink strength (Péron, 2010). Finally, necrotic tubercles were observed on every genotype (data not shown). However, necrosis appeared to be due to nutritional competition between established tubercles rather than to a true resistance mechanism. Accordingly, necrosis was rare and occurred early on (26 dpi) and no significant differences in frequency or appearance date were observed among the 10 studied WOSR genotypes. All together, these results confirmed the resistance of the genotype Darmor while Yudal, previously considered as a "susceptible" genotype, turns out to be not the most susceptible one. Despite no genotype was identified showing a higher resistance than Darmor, mini-rhizotron experiments were a good strategy for screening WOSR sources of resistance and for identifying resistance mechanisms. The present study suggests that WOSR resistance to *P. ramosa* parasitisation is a multiple component process that is expressed at different stages of the interaction, including parasite seed germination, tubercle growth and production and post-attachment stages of parasite development. Pot experiments revealed different responses to parasite infestation in four tested genotypes compared to the mini-rhizotron experiments. While the total number of tubercles on Campo, Yudal, Shakira and Grizzly differed in the mini-rhizotron experiments, these differences were not observed in the pot experiments where the total number of recovered tubercles was not significantly different. One hypothesis would be that the first resistance component of WOSR—based on a low or moderate stimulation of parasite seed germination—was "suppressed" in pot experiments. Broomrape germination in soil depends on the quantity and/or quality of the germination stimulants in the rhizosphere. Strigolactones are known to be the major stimulating substances produced from most plants (Yoneyama et al., 2010) and a recent study suggested that *B. napus*, as in the Brassicaceae family (Goldwasser et al., 2008), may produce these compounds in very low quantities (Auger et al., 2012). Some studies have also indicated that isothiocyanates (ITC), which are degradation products of glucosinolates secreted by oilseed rape roots in the soil (Virtue et al., 2006), also trigger *P. ramosa* seed germination and act as prevalent germination stimulants in the *B. napus* rhizosphere, both in the field and in the greenhouse (Auger et al., 2012). Therefore, depending on the co-culture conditions, the capacity of oilseed rape to induce parasite seed germination may strongly depend on the production of ITC. In the *B. napus* rhizosphere, ITC are generated from the hydrolysis of glucosinolates in the presence of myrosinase produced by local microbes and/or by the plant in response to root wounding. Consequently, ITC production is promoted in both field and pot conditions, but probably hampered in mini-rhizotron conditions due to limited root wounding and the lack of microflora. Therefore, under mini-rhizotron conditions, parasite seed germination may rely solely on the production of strigolactones in a genotype-dependant manner, whereas ITC is produced in pot conditions, thereby bypassing the first resistance mechanism associated with strigolactone production. Furthermore, WOSR susceptibility was significantly decreased in pot experiments using sterilised soil (data not shown), further indicating that ITC act in soil as major stimulants of WOSR through microflora activity. These findings have major consequences for breeding strategies in WOSR for resistance to *P. ramosa*. Resistance based on low germination stimulant production, as proposed for other crops such as rice and pea (Gomez-Roldan et al., 2008; Umehara et al., 2008), would be difficult to implement in WOSR due to the multifactorial character of this trait.

Nevertheless, pot experiments reveal the implication of resistance mechanisms. First, infection on the genotype Darmor remained significantly reduced compared to the other genotypes suggesting that this genotype is partially resistant to broomrape infection due to a low production of germination stimulants and/or barriers limiting parasite attachment. Second, emergence of *P. ramosa* plants on Shakira occurred several days later than on the other genotypes, indicating that tubercle growth was retarded—as observed during the mini-rhizotron experiments. Moreover, the peculiar parasite phenotype on this WOSR genotype was also observed in the pot experiments, suggesting that the traits that cause parasite developmental disorders should be investigated in *B. napus*.

In the present paper, we demonstrated that mini-rhizotron experiments can be used (albeit interpreted with precaution concerning the germination stage) for *B. napus* and other glucosinolate-producing host plants (including *A. thaliana*) to study the potential components of resistance to *P. ramosa*. Among the screened WOSR lines, we observed two resistance mechanisms occurring after parasitic seed germination: one limited the occurrence of tubercles on host roots, and one limited tubercle development. Their combination in a single genotype and subsequent evaluation of resistance are currently underway. Because to date no strong resistance has been observed among elite WOSR lines, wide screening of *B. napus* and germplasms from parental and other close species may be required to strengthen resistance and assist characterisation. Comparison of field response as shown in Table 1 and results from laboratory experiments (mini-rhizotron and greenhouse conditions) of the studied genotypes suggest that the good performance of some lines in the field, such as Shakira, may be associated with the resistance mechanisms revealed here. Interestingly, the two genotypes Grizzly and Cooper, shown to be highly susceptible in controlled conditions, show divergent response in the field. Because Grizzly performs well under field infestation in comparison with the genotype Cooper, it would be interesting to measure their level of infection in field conditions. In case of similar and strong infection for both genotypes, the presence of tolerance traits in these elite lines should be considered in breeding programs in addition to resistance traits.

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