



Brief Report

Development of Pea Breeding Lines with Resistance to *Orobanche crenata* Derived from Pea Landraces and Wild *Pisum* spp.

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Abstract: Pea (*Pisum sativum*) is an important grain legume worldwide whose cultivation is severely constrained by the root parasitic weed crenate broomrape (*Orobanche crenata*), which is widespread in the Mediterranean Basin and Middle East. No resistance is available in commercialized cultivars but some levels of incomplete resistance has been reported in pea landraces and *Pisum* spp. relatives. In this paper we report the development of a number of advanced pea breeding lines with resistance derived from wide crosses with resistant *P. fulvum*, *P. sativum* ssp. *elatius*, *P. sativum* ssp. *syriacum*, and with pea landraces, and critically discuss current progress and future perspectives on pea breeding for broomrape resistance. Resistance of breeding lines was confirmed over five field trials, showing markedly reduced broomrape over ground emergence, and in rhizotron experiments, showing either reduced tubercle formation or, in some of the lines, also hampered tubercle development that might grow slower or even become necrotic and die. Breeding lines performed well agronomically, having similar or mostly higher yields than the parental pea cv. Messire in environments with high broomrape incidence.

Keywords: pea; broomrape; parasitic weeds; resistance; breeding



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1. Introduction

Pea (*Pisum sativum* L.) is a cool season legume grown worldwide as a source of protein both for human food and animal feed. Significant efforts have been made in pea breeding for disease resistance in continental and oceanic conditions where it is mainly spring sown [1]. Relative prevalence and importance of the various diseases varies with the agroecological conditions. The root parasitic weed broomrape (*Orobanche crenata* Forsk.) is widespread in the Mediterranean Basin and Middle East, and with climate change, it is spreading further north in Europe, and further south in Africa [2]. In this area broomrape is the major constraint for pea production [3,4]. So far, the effectiveness of conventional control methods is limited due to numerous factors, in particular the complex nature of the parasite, which reproduces by tiny and long-living seeds [5].

Breeding for broomrape resistance is difficult considering the scarce and complex nature of resistance in legumes in general [6] and in pea in particular. Only incomplete resistance to *O. crenata* was found in other grain legumes such as faba bean (*Vicia faba* L.), which has been successfully accumulated by breeding, allowing the release of resistant cultivars [6–8]. A similar effort on pea breeding for broomrape resistance has only recently been started with no resistance available in any pea cultivar or breeding line by that time [3], but various levels of resistance reported in germplasm accessions of *P. sativum* ssp. *sativum*, *abyssinicum*, *arvense*, and *elatius*, and in *Pisum fulvum* Sibth. & Sm. [9–11]. Resistance to *O. crenata* in pea has been reported to be quantitative and polygenic [12] and highly influenced by the environment, complicating the selection of the most resistant genotypes. Identified resistance to *O. crenata* in pea has been characterized at various

levels from histology to proteomics [9,12–14], but underlying gen(es) are still unknown. The rapid development of pea genomic tools will certainly facilitate characterization and use in breeding of the still undiscovered resistance genes. Meanwhile, we progressed in crossing and selection under field conditions and succeeded in development of a number of advanced breeding lines resistant to broomrape infection that are described here.

2. Materials and Methods

The pea network described here consisted of 9 pea breeding lines derived from the IAS-CSIC pea breeding program [11]. In brief, crosses were made among (partially) resistant accessions (*P. sativum* ssp. *sativum* Ps624 and Ps423, *P. sativum* ssp. *elatius* P675, *P. sativum* ssp. *syriacum* P665, and *P. fulvum* P660) [9–11] and pea cultivar Messire and others, and submitted to pedigree yearly selection from F₂. The lines described here include selection from single crosses (GC number), backcrosses (BC number), or multiple crosses (GCC number). At this stage of the breeding program, the first priority in selection was broomrape resistance, with agronomic performance being second. Taking into consideration that progenies originated from wide crosses, fertility, growth habit and other yield-related traits were low in early generations. Basically, within the most broomrape-resistant progenies, the ones that were most fertile and had better agronomical potential were selected, discarding the rest. As a result, 9 resistant and productive breeding lines were selected after 6 seasons of yearly selection under heavy and uniform *O. crenata* soil infestation conditions. Their performance was further characterized in the field in Córdoba (Spain) during three consecutive seasons and at Escacena del Campo (Huelva, Spain) during two seasons, and under controlled conditions using mini-rhizotrons.

A randomized complete block design with three blocks was used in all field studies. Pea cv. Messire, frequently used in early crosses and highly susceptible to *O. crenata*, was included as a check in all experiments. Each replicate consisted of 1 m² plots consisting of 3 rows 1 m long, separated by 0.33 m, with 10 plants per row. Sowings were carried out between December and January, according to local practice. At the end of the crop cycle, the number of emerged *O. crenata* shoots per pea plant (Oc/pl, Table 1) was scored by counting the total number of pea plants and the total number of emerged *O. crenata* shoots per plot. At plant maturity the plots were harvested. Grain yield was assessed by weighing the seeds produced per plot and estimating the corresponding grain yield (kg/ha).

The in vitro studies were performed using mini-rhizotrons as earlier described [9,15]. Pea seeds were surface sterilized with 2% (w/v) NaOCl solution and 0.02% (v/v) Tween 20 for 5 min and then rinsed thoroughly with sterile distilled water and germinated for 4 days in 9 cm diameter Petri dishes with moistened filter papers at 23 °C conditions. Three pea seedlings were individually transferred to glass fiber filter paper (GFFP) (Whatman International Ltd., Maidstone, UK) sheets and placed over square Petri dishes (12 cm by 12 cm) filled with sterile perlite moistened with sterile distilled water. The Petri dishes were previously punctured on the top to allow pea stem develop outside of the dish. The seeds of *O. crenata* were surface sterilized by immersion in 0.5% (w/v) NaOCl and 0.02% (v/v) Tween 20 for 5 min, rinsed thoroughly with sterile distilled water, and spread separately over the GFFP sheets at a density of 50 seeds per cm². The Petri dishes containing the pea–*O. crenata* co-cultivation system were sealed with parafilm, wrapped in aluminum foil, and stored vertically in a growth chamber (23/20 °C, 16/8 h day/night). The plants received Hoagland's nutrient solution modified at one-quarter strength once per week. *O. crenata* seeds located at a distance of 3 mm from the pea roots were inspected under a stereoscopic microscope (Leica S9i, Leica Microsystems GmbH, Wetzlar, Germany) to determine (i) the percent of contacted *O. crenata* radicles that successfully penetrated pea roots and formed a healthy tubercle at 30 days after infection (dai), (ii) the percent of total formed tubercles that became necrotic and died by 45 dai, and (iii) the developmental stage of *O. crenata* tubercles at 30 and 45 dai: T1: tubercle with a diameter <1 mm without crown roots, T2: tubercle with a diameter >1 mm without crown roots, T3: formation of crown roots, and T4: formation of shoot [9].

Table 1. Field response of pea breeding lines to *Orobanche crenata* compared to the parental cv. Messire.

| Line | Pedigree | FC/LT | Field Studies | | | | | | | | | |
|------------|---------------------------------|-------|---------------|----------|----------|---------|----------|-------|----------|-------|-------|--------|
| | | | Cor07 | | Cor08 | | Cor09 | | Esc08 | | Esc09 | |
| | | | Oc/pl | Kg/ha | Oc/pl | Kg/ha | IOc/pl | Kg/ha | Oc/pl | Kg/ha | Oc/pl | Kg/ha |
| Messire | Check cv | WF/NL | 3.50 | 1051 | 3.89 | 491 | 1.54 | 1264 | 1.83 | - | 0.02 | 1537 |
| GC248-NS46 | Ps624/Messire | WF/NL | 0.13 *** | 1638 | 0.43 ** | 3002 ** | 0.15 ** | 709 | 0.17 *** | - | 0.00 | 640 ** |
| GC233-J5 | Ps565/Ps624 | WF/NL | 0.13 *** | 3417 *** | 0.59 ** | 1934 * | 0.18 ** | 1517 | 0.07 *** | - | 0.00 | 1338 |
| GCC136-J24 | Ps624/Ps423/Radley | WF/AT | 0.23 *** | 2411 * | 1.44 * | 804 | 0.18 ** | 388 * | 1.03 * | - | 0.03 | 568 ** |
| BC20-J10 | Messire/P660 | WF/NL | 0.17 *** | 1003 | 0.51 ** | 2493 ** | 0.12 ** | 719 | 0.08 *** | - | 0.00 | 735 |
| BC20-J11 | Messire/P660 | WF/NL | 0.20 *** | 3456 *** | 0.54 ** | 1643 | 0.08 ** | 697 | 0.13 *** | - | 0.01 | 998 |
| BC20-J13 | Messire/P660 | WF/NL | 0.27 *** | 1955 | 0.70 ** | 2431 ** | 0.20 ** | 765 | 0.17 *** | - | 0.00 | 1251 |
| BC20-J15 | Messire/P660 | WF/NL | 0.17 *** | 2077 | 0.51 ** | 1854 * | 0.21 ** | 690 | 0.17 *** | - | 0.00 | 855 * |
| GCC99-J17 | P675/P665/J11760/Messire/Ballet | CF/AT | 0.07 *** | 1671 | 0.39 *** | 1472 | 0.06 *** | 54 | 0.07 *** | - | 0.01 | 514 ** |
| GCC124-J19 | Messire/P660/Ballet | CF/NL | 0.20 *** | 3579 *** | 0.47 ** | 1855 * | 0.26 ** | 918 | 0.10 *** | - | 0.03 | 1533 |

Footnote: FC = flower color (WF = white; CF = colored); LT = Leaf Type (NL = normal leaf; AT = aphylla type); Oc/pl = number of *O. crenata* emerged per pea plant; kg/ha = grain yield. *, **, *** indicates significant differences (LSD) at $p < 0.05$, 0.01, and 0.001, respectively, compared to the Messire check.

3. Results

The focus of the yearly selection was on *O. crenata* resistance. As a result, selected progenies showed reduced *O. crenata* infection levels compared to the Messire pea cultivar used as the control (Table 1). The level of *O. crenata* infection varied among environments, being high in Córdoba during the 2006–2007 season (Cor07) and 2007–2008 season (Cor08), with 3.5 and 3.89 *O. crenata* emerged per Messire plant (Oc/pl), respectively; moderate in Córdoba during the 2008–2009 season (Cor09) and Escacena during 2007–2008 (Esc08), with 1.54 and 1.83 Oc/pl, respectively; and negligible in Escacena during the 2008–2009 season (Esc09). *O. crenata* infection on all selected pea breeding lines was significantly and markedly lower than on Messire, in the range of 0.07–0.27 Oc/pl ($>13 \times$ fold reduction compared to Messire) at Cor07, in the range of 0.39–1.44 Oc/pl ($>3 \times$ fold reduction) at Cor08, and in the range of 0.06–0.25 Oc/pl ($>6 \times$ fold reduction) at Cor09, and of 0.07–0.91 Oc/pl ($>2 \times$ fold reduction) at Esc08.

Pea selections overyielded cv. Messire in the environments with high *O. crenata* infection (Cor07 and Cor08), however, yields were similar or even lower than Messire when *O. crenata* pressure was low (Cor09 and Esc09). All pea selections resembled parental Messire in flower color and plant morphology except GCC99-J17, which still had colored flowers from wild parents, and the aphylla type, a trait probably inherited from the Ballet parent. Breeding line GCC136-J24 also showed the aphylla type, conferred from the Radley parent.

Rhizotron studies confirmed the observed field resistance of the selected breeding lines (Table 2). Over 71% of the *O. crenata* seedlings successfully penetrated the roots of Messire and formed a tubercle, which developed fast and healthy on Messire roots, being already in the spider stage with formation of crown roots (stage T3) by 30 dai and in the stage of shoot development (stage T4) by 45 dai, none of them being necrotic. The capacity of *O. crenata* radicles to penetrate the pea roots and form tubercles was significantly reduced in all breeding lines, being in the range of 0.5–34.7% ($>2 \times$ fold reduction compared to Messire). In addition to resistance reducing tubercle formation, some breeding lines exhibited additional resistance hampering tubercle development. Those tubercles formed on the roots of GC248-NS46, BC20-J13, BC20-J15, and GCC124-J19, which developed more slowly than on Messire roots. The necrosis of *O. crenata* tubercles formed on BC20-J10 and GCC124-J19 was visible at 45 dai (50 to 100%, respectively).

Table 2. Rhizotron study of the responses of pea breeding lines to *Orobanche crenata* compared to the parental cv. Messire.

| Genotype | In Vitro Study | | | |
|------------|----------------|-----------------------|------------------------------|--------|
| | Oc Tubercle(%) | Tubercle Necrosis (%) | Tubercle Developmental Stage | |
| | 30 dai | 45 dai | 30 dai | 45 dai |
| Messire | 71.1 | 0.0 | T2–T3 | T3–T4 |
| GC248-NS46 | 34.7 ** | 0.0 | T1–T3 | T2–T3 |
| GC233-J5 | 31.2 *** | 0.0 | T1–T3 | T3–T4 |
| GCC136-J24 | 12.5 *** | 0.0 | T1–T3 | T3–T4 |
| BC20-J10 | 29.2 ** | 0.0 | T2–T3 | T3–T4 |
| BC20-J11 | 25.3 *** | 50.0 * | T1–T2 | T2–T4 |
| BC20-J13 | 30.6 ** | 0.0 | T1–T3 | T2–T3 |
| BC20-J15 | 0.5 *** | 0.0 | T2 | T2 |
| GCC99-J17 | 15.6 *** | 0.0 | T1–T3 | T3–T4 |
| GCC124-J19 | 8.3 *** | 100.0 *** | T3 | T3 |

Footnote: the percent of contacted *O. crenata* radicles that successfully formed tubercles at 30 days after inoculation (dai), the percent of tubercles that necrosed at 45 dai, and the developmental stage of the tubercles (T1: tubercle with a diameter <1 mm without crown roots, T2: tubercle with a diameter >1 mm without crown roots, T3: formation of crown roots, and T4: formation of shoot) at 30 and 45 dai. *, **, *** indicate significant differences (LSD) at $p < 0.05$, 0.01, and 0.001, respectively, compared to the Messire check.

4. Discussion

Reduced levels of *O. crenata* infection were successfully transferred from landraces of *P. sativum* ssp. *sativum* (Ps624, Ps423), *P. sativum* ssp. *syriacum* (P665), *P. sativum* ssp. *elatius* (P675), and *P. fulvum* (P660) to the adapted breeding lines described here. The resistance achieved was not complete, but it was at the levels of the donors and of other breeding lines [9,10,15,16].

Resistance against root parasitic weeds is a multicomponent event, being the result of a battery of avoidance factors, resistance mechanisms acting at different levels of the infection process, or both [11,17,18]. Rhizotron experiments confirmed that the reduced *O. crenata* infection in the pea breeding lines observed in the field was not the result of escape or avoidance as reported in some legumes [18,19], but rather was due to true genetic resistance preventing *O. crenata* tubercle formation and development. True resistance in pea has also been reported acting before haustoria development by reducing the induction of *O. crenata* seed germination [9,20]. However, this was not studied here since the roots of the donors of resistance of the described breeding lines induced levels of *O. crenata* germination at the level of susceptible lines [15]. Pre-haustorial mechanisms of resistance preventing *Orobanche* tubercle formation and post-haustorial ones hampering development of formed tubercles have been described in pea and a range of crops [9,15,17,20–26].

The current focus in applied breeding is leveraging biotechnological tools to develop more and better markers to allow marker-assisted selection with the hope that this speeds up the delivery of improved cultivars to the farmer. A previous study on a Recombinant Inbred Line population derived from the cross P665 *Messire shows a quantitative inheritance governed by several QTLs [12]. These were of rather small effect that precluded the development of markers to be used in Marker Assisted Selection (MAS). However, by classical field selection we succeeded in selecting a breeding line (GCC99-J17) with resistance derived from this P665 together with Pe675, reinforcing the value of classical breeding that allowed us to achieve some relevant progress, even in absence of the most wanted availability of molecular markers. The situation was similar for faba bean, in which a number of studies reported quantitative inheritance governed by a number of QTLs explaining rather little phenotypic variation, and therefore not yet used in MAS [27], which has retarded but not prevented the efficient breeding and release of resistant cultivars [6,7]. No genetic studies have ever been performed to unravel the inheritance of the resistance of Ps624 or

Pf660. Based on current information, we cannot conclude on their inheritance. However, the fact that we succeeded in selecting resistance from Ps624 in progenies derived from a single cross, and from P660 in progenies of backcrosses with Messire or multiple crosses with other elite cultivars, allows us to speculate that inheritance of *O. crenata* resistance in pea might not be as complex as earlier suggested [11,12], and that efficient field selection is feasible.

The key to the development of cultivars with long-lasting broomrape resistance is diversity [8]. A first strategy could be pyramiding more than one gene or by combining monogenic and polygenic resistance with the help of reliable molecular markers for efficient selection. It is widely acknowledged that complex resistance is likely to be more durable than monogenic resistance. However, the durability of resistance is not solely an issue of the number of genes, but also depends on the mechanism of action of the genes. Rather than pyramiding several genes acting at the same level, combining different resistance mechanisms providing multiple barriers not easily overcome by parasite changes may provide more durable outcomes. For instance, the “low germination induction” resistance, likely to be singly inherited [8,20], could be most relevant for providing a durable outcome, particularly when used in combination with other mechanisms, such as the ones reported in these new breeding lines.

The accelerated progress in the genomic and biotechnological research faced with pea, with a genome sequence recently released [28], will soon facilitate the understanding of crucial developmental mechanisms in both the parasite and the host and will speed gene discovery and the development of breeder-friendly molecular markers [8]. Unfortunately, less is yet understood of *O. crenata* genomics, with some relevant info on related species such as *Phelipanche aegyptiaca* [29] and *O. cumana* [30] that might help in understanding parasite virulence and host resistance mechanisms. This will facilitate gene discovery that will accelerate breeding through the development of markers for efficient MAS, as already done in sunflower breeding for *O. cumana* resistance [31,32], or allow genome editing. Unfortunately, this is not yet available for pea/broomrape. Meanwhile, we progressed in identifying a range of sources of resistance and in introducing them into an adapted pea background through sexual crossing and yearly field selection.

The result is the development of a number of advanced pea breeding lines resistant to broomrape infection that are described here and that are available on request to be used as parents in breeding programs elsewhere. Selections described here overyielded the parent pea cultivar Messire when the *O. crenata* infection was high, although yields could be lower in the absence of *O. crenata* pressure. However, standing ability should still be improved.

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