INOCULATION AND GROWTH WITH ROOT PARASITIC WEEDS

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ABSTRACT

A number of parasitic plants have become weeds, posing severe constraints to major crops including grain legumes. Breeding for resistance is acknowledged as the major component of an integrated control strategy. However, resistance against most parasitic weeds is difficult to access, scarce, of complex nature and of low heritability, making breeding for resistance a difficult task. Only moderate to low levels of incomplete resistance of complex inheritance has been identified against most *Orobanche* species. This has made selection more difficult and has slowed down the breeding process. Resistance is a multicomponent event, being the result of a battery of escape factors or resistance mechanisms acting at different levels of the infection process. Understanding these mechanisms will help to detect existing genetic diversity for mechanisms that hamper infection. The use of *Medicago truncatula* as a model to study the resistance and pathogenesis of parasitic weeds will allow a better knowledge of those mechanisms.

INTRODUCTION

Over 4,000 species of angiosperms are able to directly invade and parasitize other plants (Nickrent et al., 1998). Unfortunately for farmers, a small number of these species have become weeds, posing severe constraints to major crops including grain legumes. By far the most economically damaging are root parasites belonging to the genera *Orobanche* (broomrapes) and *Striga* (witchweeds). *Orobanche* species, found largely in Mediterranean and warm temperate areas of Europe, North Africa and the Middle East are holoparasites, devoid of chlorophyll and totally dependent on the host for organic carbon, water and nitrogen. *Striga* and the closely related genus *Alectra* are hemi-parasitic and a particular problem in sub-humid and semi-arid areas of Africa (Parker and Riches, 1993; Joel et al., 2007).

The weedy root parasites, which are often host specific, exert their greatest damage prior to their emergence; therefore the majority of field loss may occur before diagnosis of infection. In spite of intense efforts during the 20th century, effective means to selectively control the various species of parasitic weeds are still scarce or lacking. A wide variety of approaches - physical, cultural, chemical and biological - have been explored against root parasites, but most of them are not effective, or not selective to the majority of susceptible crops.

O. crenata (crenate broomrape) has been known to threaten legume crops since antiquity (Cubero et al., 1994). It is an important pest in faba bean (*Vicia faba*), pea (*Pisum sativum*), lentil (*Lens culinaris*), vetches (*Vicia* spp.), grass pea (*Lathyrus sativus*) and other grain and forage legumes in the Mediterranean Basin and Middle East (Rubiales et al., 2006). Typically there is a low genetic differentiation among *O. crenata* populations in the Mediterranean Area and a considerably high variation within each population, which is consistent with the predominantly allogamous behaviour of *O. crenata* (Román et al. 2002). This species occurs exclusively in agricultural and disturbed habitats, and is highly adapted to agricultural conditions.

O. aegyptiaca (Egyptian broomrape) is an important pest of many crops in the Middle East and Asia. Similar to *O. crenata* this species attacks faba bean, common vetch, grasspea, chickpea and lentil, but in addition it can also severely attack peanut (*Arachis hypogea*). Cruciferous crops, particularly cabbage and oilseed rape, and several members of the families Solanaceae, Apiaceae and Asteraceae are also susceptible to *O. aegyptiaca* (Parker and Riches, 1993).

O. foetida is widely distributed in natural habitats in the Western Mediterranean Area parasitising wild herbaceous leguminous plants of the genera *Anthyllis, Astragalus, Ebenus, Lotus, Medicago, Ononis, Scorpiurus* and *Trifolium* (Pujadas-Salvá, 2002). It is, however, considered an important agricultural parasite in faba bean in Beja region of Tunisia (Kharrat et al., 1992). Of the cool-season grain legumes only pea (*Pisum sativum*) escapes its attack (Kharrat, 1999). Infection by this broomrape has also been reported in Tunisia on *M. truncatula* (Kharrat, 2002). It has recently also been found in Taounate, Morocco infecting common vetch (Rubiales et al., 2005b).

Although other *Orobanche* species can infect leguminous plants, they are generally of little economic importance. *O. minor*, however, has a wide host range among forage legumes in temperate climates. It resembles *O. crenata* in its stout, unbranched stem, but it has smaller flowers, with smaller lips. It was known in the past as an agricultural weed in

Europe, but currently it is not common as such any more. It is found in native and disturbed habitats throughout the central and southern parts of Europe, and extends to the eastern coast of Africa and southwards (Parker and Riches, 1993). In addition, it was imported to various other parts of the world and is currently found as a garden weed. It is of economic importance on clover (*Trifolium* spp.) that is grown for seed and has recently become a problem on red clover in Oregon, USA (Osterbauer and Rehms, 2002; Eizenberg et al., 2004).

Orobanche ramosa attacks many different crop plants; its host range resembles that of *O. aegyptiaca*. Its host range is very wide, attacking potato, tomato, eggplant, tobacco, brassicas, melon, watermelon, cucumbers, and to a lesser extent parsley, celery, parsnip and lettuce. *O. ramosa* can infect several legumes, but not as an agricultural problem. However, it is interesting that it can infect *M. truncatula*.

Striga gesnerioides and *Alectra vogelii* cause considerable yield reduction of grain legume crops, particularly cowpea (*Vigna unguiculata*), throughout semi-arid areas of sub-Saharan Africa (Parker and Riches, 1993). *S. gesnerioides* occurs in natural vegetation throughout the drier regions of Africa parasitizing several species of Leguminosae and Convolvulaceae. *A. vogelii* infects a number of grain legumes, mainly cowpea, soyabean in south and central Africa.

Breeding for resistance is the most economic, feasible and environmental friendly method of control. However, appropriate screening methods and effective selection indices are needed to ensure success. Resistance against most parasitic weeds is difficult to access, scarce, of complex nature and of low heritability, making breeding for resistance a difficult task. In spite of these difficulties, significant success has been achieved in some crops. In a few instances, resistance of simple inheritance has been identified and widely exploited in breeding. This has been particularly important allowing rapid progress to develop cultivars of sunflower that are resistant to *O. cumana* and of cowpea resistant to *S. gesnerioides*. However, breeding programmes based on only a few dominant genes are in serious risk of breakdown of resistance. A well studied case is *O. cumana* attacking sunflower in which at least seven races have so far been described (Fernández-Martínez et al., 2005).

Host plants might escape infection by reduced root biomass and by root architecture that avoids the soil layer in which the seeds of the parasite are more common. The host may limit damage (tolerance) by factors that influence source–sink relationships, such as osmotic pressure (Wegmann et al., 1991). Low germination stimulant production has been successfully exploited in breeding sorghum cultivars that are resistant to *Striga asiatica* (Ejeta et al., 2000). Although low induction of germination was considered to play little role in resistance to *Orobanche* in legumes (terBorg, 1999), this trait has recently been found in accessions of a range of legumes (Rubiales et al., 2006).

Understanding the escape and resistance factors will help to detect existing genetic diversity for mechanisms that hamper infection (Labrousse et al., 2001; Rubiales et al., 2003c). Combining different resistance mechanisms into a single cultivar will provide a durable outcome. This can be facilitated by the use of *in vitro* screening methods that allow the identification of highly heritable resistance components, together with adoption of marker-assisted selection techniques (Ouedraogo et al., 2002; Román et al., 2002a; Pérez-Vich et al., 2004; Valderrama et al., 2004).

The excellent traits of *Medicago truncatula* as a model plant make it also a suitable model for pathogenic interactions. In the case of parasitic plants, the main handicap is to get a collection of susceptible and resistant genotypes to the pathogen. To date, susceptible

and resistant accessions have been identified mainly against *O. crenata* (Rodríguez-Conde et al., 2004), but our working groups have also tested and identified different resistant and susceptible accessions to other broomrape species like *O. aegyptiaca*, *O. cumana*, *O. minor* and *O. ramosa* among others (Fernández-Aparicio, unpublished results).

Techniques of screening
Resistance indices
One of the problems in breeding for broomrape resistance is the lack of an effective selection criterion and of a suitable screening method. Several indices can be used to measure the levels of resistance to parasitic weeds, such as height of the parasitic flowering shoots, total weight of shoots per host plant, number of emerged shoots per unit area, rate of reproduction, etc., but the most widely used index for resistance to *Orobanche, Alectra* and *Striga* is the total number of emerged shoots per host plant (Gil et al., 1987; Rubiales et al., 2006). *Orobanche* attack is related to the growth vigour of the host and there is a competition for resources among attachments (Aalders and Pieters, 1987), thus, indices based on size and weight of broomrapes can be misleading.

Field testing
Field trials with a large number of accessions are generally used by breeding programmes to select potentially resistant genotypes from a germplasm collection (Rubiales et al., 2002, 2003a). Trials are undertaken under field conditions using heavily infested plots. It is crucial to ensure uniform distribution of the parasite seeds in the soil to prevent selection of genotypes that merely remain unchallenged. For small scale tests, the plots can be artificially inoculated mixing parasite seeds with sand and applying them to the row with the crop seeds when sowing. However, escapes cannot be precluded, and each test row should be surrounded by rows of a susceptible and vigorous check to be used as a reference for each accession (Rubiales et al., 2003a).

Pot testing
Pot methods allow control over the environment, the inoculum density and its origin. Several methodologies (substrate, pot size, etc.) can be used. Linke et al. (1991) suggested the use of plastic pot (5 l) with steam sterilized soil-sand (3:1 v:v) mixed with 7500 (about 30 mg) *O. crenata* seeds/kg substrate to screen chickpea for broomrape resistance. A mixture of clay, silt sand and organic matter (58:22:18:2%) with 40 mg seeds/kg substrate in 2 l pots has been suggested by Goldwasser et al. (1997). A mixture of vermiculite-sand (3:1 v:v) and some fertilizer, and adding 25–30 mg seeds in 1 l pots also gives good results (Pérez-de-Luque et al., 2004). It is possible to use other substrates like soil or peat instead of vermiculite, but they can hinder the later observation of the broomrape tubercles. The main point is to get a substrate that allows a good plant growth and broomrape infection, and can be easily removed and washed from the roots, facilitating observation of parasite development.

In vitro testing
Various techniques have been used to allow close observation of the germination, attachment and early development of parasitic weeds. These so called *in vitro* methods can be used to characterise the resistance of lines selected under field conditions. Use of Petri dishes was described by Sauerborn et al. (1987) for mass screenings of lentils under

controlled conditions. This method has been successfully applied with slight modifications by Rubiales et al. (2003c, 2004), Sillero et al. (2001), Pérez-de-Luque et al. (2005a) to characterise the resistance of several grain legumes, and by Linke et al. (1993) and Sillero et al. (2005) to study the resistance of forage legumes. It has also been used to characterise early stages of interaction of several *Orobanche* species with *M. truncatula* (Rodríguez-Conde et al., 2004).

Plants (a minimum of 10 per entry) are grown individually in 15 cm diameter Petri dishes with 8mg of surface-sterilized and conditioned *Orobanche* seeds spread over glass microfibre filter discs. The Petri dishes are sealed with parafilm, wrapped in aluminium foil and placed vertically, the hole with the germinating plant seed up and uncovered to allow seedling emergence and growth, in a growth chamber at 20 ∘C. Forty-five days later broomrape attachments per Petri dish are counted. Root length should be estimated in order to exclude escapes due to differing amount of roots produced per genotype. The intercept method of Tennant (1975) can be used. Number of broomrape attachments per plant should be referred to as number of attachments per root length unit.

Petri dish methods also allow more detailed studies on induction of germination and resistance to penetration. Germination can be monitored about 30 days after preparing the plates, by studying 500 seeds that are close (<3 mm) to the host roots per petri dish under a binocular microscope at 30× magnification to determine the percentage of germination. The method also allows exogenous applications of the germination stimulant GR24 to bypass the germination step and concentrate on observations of attachment or post-attachment events. The experimental procedure is the same as above, but 10 days after preparation 5ml of GR24 (10 ppm) are uniformly distributed on the filter disc. Percentage of germination is studied 7 days after GR24 application. Ten days later the broomrape radicles contacting the host roots can also be studied and the percentage of those causing a necrosis of the host tissue surrounding the contact point determined. Five days later the final number of attachments is counted and root length estimated as above.

The "sandwich" assay involves growing host and parasite in sand or vermiculite held in the gap between two glass sheets. Two cork strips of 0.5 cm thickness are placed between both glasses in left and right sides, and the lower side is sealed with a porous material (foam rubber, sponge) that allows nutrient solution to penetrate. Seeds are placed on the sand in the upper side of the plates. This method allows large plants to be grown between glass sheets of 50 cm × 30 cm, and also to follow the roots spatial distribution and the development of the broomrapes at different depths.

Pot and *in vitro* methods (either Petri dishes, polyethylene bags, glasses, etc) are faster and cheaper than the field screening, prevent the escapes due to an uneven distribution of the *Orobanche* seeds in the soil and reduce the environmental influence. Also the effect of plant vigour and root length can be more easily studied. These methods also allow more detailed studies on both the inheritance and the mechanisms of resistance. A further advantage is the feasibility of testing with different *Orobanche* populations and species.

Histological studies

Several simple techniques are available to develop histochemical studies of the interaction between parasitic plants and legumes. Usually the samples are fixed in FAA (ethanol 50% + formaldehyde 5%+glacial acetic acid 10%, in water) for 48 h, embedded in paraffin after dehydration and cut with a rotary microtome. In some cases, the fixation

procedure is changed and samples are cut by hand when it is required for the staining method.

Different cytochemical methods can be used and combined with light and fluorescence microscopy in order to identify several mechanisms of resistance described in other pathosystems. The following list does not intend to be exhaustive, and many other techniques can be developed:

(i)   Alcian green - safranin (AGS) (Joel, 1983). With this staining method, carbohydrates (including cell walls and mucilage) appeared green, yellow or blue, while lignified, cutinized and suberized walls, as well as tannin and lipid material inside cells appeared red (Joel, 1983).

(ii)   Staining with 0.05% toluidine blue O (TBO) in PO_4 buffer (pH 5.5) during 5-10 min can be also used. In this case the dye must be applied before removal of paraffin (Ruzin, 1999). This method allows the detection of phenolics as well as tannins, lignin and suberin (Baayen et al., 1996; Bordallo et al., 2002; Mellersh et al., 2002; Crews et al., 2003).

(iii)   Phloroglucinol (2% in ethanol)–HCl (35%) (Ruzin, 1999) stains the aldehyde groups of lignin and suberin, but quenches lignin autofluorescence and retains suberin fluorescence (Baayen et al., 1996; Rioux et al., 1998). So this method can be used to detect lignins and suberins combining light and fluorescence microscopy

(iv)   Aniline blue fluorochrome is used for callose detection under UV fluorescence (340-380 nm) (Bordallo et al., 2002).

(v)   Pectins can be detected using ruthenium red. Non-methyl esterified pectins take a red/pink coloration with this dye (Vallet et al., 1996).

(vi)   Protein cross-linking in cell walls can be determined following the procedure described by Mellersh et al. (2002). Fixed samples are cut by hand and submerged in 1% sodium dodecyl sulphate (SDS) for 24 h at 80ºC. They are stained for 3-5 min in 0.1% coomassie blue in 40% ethanol/10% acetic acid, rinsed in a solution of 40% ethanol/10% acetic acid and mounted in distilled water. Cell walls with protein cross-linking take a deep blue colour.

(vii)   For determination of H_2O_2 and peroxidase activity, fresh samples are stained with 3,3-diaminobenzidine (DAB) using a modification of the procedure described by Thordal-Christensen et al. (1997). Fresh samples are submerged in DAB solution (1 mg·ml^{-1}, pH 3,8) in distilled water for 2-3 hours. After that, the samples are washed with lactic acid, glycerol and water (1:1:1) for an hour, hand cut with a razor blade and mounted on slides with lactic acid, glycerol and water (1:1:1).

(viii) Accumulation of phenolic compounds can be determined on fresh hand cut samples observed under epi-fluorescence (340-380 nm).

It is important to notice that some histochemical methods alone are not sufficient to conclude that a mechanism of resistance is taking place, and some of them are also more reliable than others. For that reason it is advisable to resort to some different methods to check the same phenomenon. For example, in case of accumulation of phenolic compounds it is convenient to use fluorescence observations under UV and not only TBO staining, detecting lignin and suberin can be afforded combining Phloroglucinol staining and observations under polarized light, etc.

The new molecular approaches will serve to assist legume breeders and geneticists in identifying promising resistant genotypes and will facilitate the efficient transfer of the resistance genes among breeding lines. Furthermore, molecular markers linked to broomrape resistance genes will provide a better understanding of the genetic basis of resistance against parasitic weeds. The development of new techniques in the field of histology is allowing more reliable and precise observations, in some cases also using fresh and untreated samples, as with confocal laser scanning microscopy (CLSM). The development and application of new techniques for cytological and cytochemical research will open new gates to our understanding of the resistance process to parasitic plants. Some of these techniques are being recently adapted from human and animal investigation to plant research, and most of the possibilities for the plant sciences are yet to be developed.

Confocal laser scanning microscopy (CLSM) can provide very useful information allowing the observation of fluorescent metabolites in cells from fresh material, for example phenolic compounds, and 3D reconstruction of samples. A great advantage of this technique is that optical sectioning of samples avoid lost and destruction of difficult and scarce material during normal physical sectioning. In fact, this technique has already been used successfully for detecting phenolic compounds during the incompatible interaction between *O. cumana* and sunflower (Echevarría-Zomeño et al., 2006). CLSM can also be easily used for immunocytology employing fluorescent conjugated antibodies, as has been shown with *O. crenata* and *Vicia sativa* (Pérez-de-Luque et al., 2006b). Another important application of CLSM is for fluorescent *in situ* hybridization (FISH): in the case of the interaction of *O. crenata* with pea, FISH has been successfully used to study the expression of two genes (a peroxidase and a glucanase) implicated in the resistance to *O. crenata* (Pérez-de-Luque et al., 2006a).

The utilization of FISH for gene expression studies is part of a new branch of the histology named molecular histology, which joins molecular techniques with histological studies, and represents a promising way for further understanding of plants processes by increasing the resolution of analyses to tissue- or even cell-specific level. FISH with mRNA allows the study of genes expressed under certain conditions and within specific cells and tissues as mentioned above. On the other hand, laser capture microdissection (LCM) allows collection and isolation of specific tissues or cell groups for further molecular analysis of large number of genes (microarrays, RT-PCR, proteomic, etc.), being this the most recently and reliable adapted technique to plant research (Asano et al., 2002; Nakazono et al., 2003; Casson et al., 2005) and plant-pathogen interactions (Ramsay et al., 2004).

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