Medicago truncatula AS A MODEL FOR STUDYING INTERACTIONS BETWEEN ROOT PARASITIC PLANTS AND LEGUMES

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ABSTRACT

Parasitic angiosperms are a pest for many crops in the World. Broomrapes (*Orobanche* spp.) belong to this group of parasites and attach to the roots of the hosts in order to take water and nutrients from them. This causes a pernicious effect on the crops which can lead to a complete yield loss in some cases. No complete control measures are available to date, but breeding for resistance remains as one of the most feasible and environmental friendly methods. However, the mechanisms governing the interaction between these parasites and the host are yet not well understood, including those responsible for resistance. For that reason, the development of model plants to study these interactions will allow a better knowledge of those mechanisms. In the present work, the use of *Medicago truncatula* as a model to study the resistance and pathogenesis of *Orobanche* spp. will be presented, and different techniques available for those studies (histology, gene expression, proteomic) will be discussed.

INTRODUCTION

- **Parasitic plants and crops**

One per cent of angiosperms (about 4000 species) are either hemi- or holoparasitic plants (Press & Graves, 1995). They represent a fascinating group of plants of great interest for botanists and ecologists (Kuit, 1969): these include very beautiful curiosities like *Rafflesia* spp. (with the biggest flowers in the world) and some others more familiar like mistletoes (*Viscum* spp.). Unfortunately for farmers, some of them have adapted to grow on important crops like cereals, solanaceae and legumes with devastating effects. This is the case of the genera *Striga*, *Orobanche* and *Cuscuta*. We will focus on parasitic plants

infecting legumes, specifically *Orobanche* spp. (broomrapes), but for a better knowledge of other genera refer to Parker & Riches (1993), Press & Graves (1995) and Joel et al. (2006).

Orobanche spp. are found largely in Mediterranean and warm temperate areas of Europe, North Africa and the Middle East. They are holoparasites, devoid of chlorophyll and totally dependant on the host for organic carbon, water and nitrogen (Joel et al., 2006). Some species are specialized in parasitizing a few group of plants or species, like *O. cumana* on sunflower (*Helianthus annuus*) or *O. crenata* on legumes. Others, on the contrary, have a broad spectrum of hosts as it is the case of *O. aegyptiaca* and *O. ramosa* (Parker & Riches, 1993; Press & Graves, 1995).

O. crenata (crenate broomrape) has been known to threaten legumes crops since antiquity (Cubero & Moreno, 1996). It is an important pest in faba bean (*Vicia faba*), pea (*Pisum sativum*), lentil (*Lens culinaris*), vetches (*Vicia* spp.), grass and chickling pea (*Lathyrus sativus* and *L. cicera*) and other grain and forage legumes in the Mediterranean basin and Middle East (Sauerborn, 1991; Rubiales, 2001). Yield loss vary depending on the host crop: they can reach almost the complete loss of the crop in cases like pea (Bernhard et al., 1998; Rubiales et al., 2003a). In faba bean and lentil, losses from 5 to 95% have been reported (Mesa-García & García-Torres, 1986; Bayaa et al., 2000; Sauerborn, 1991) depending on the infestation level and the planting date.

In the Middle East and Asia, *O. aegyptiaca* (Egyptian broomrape) attacks faba bean, common vetch, chickpea and lentil, but in addition it can also attack peanut (*Arachis hypogea*), cruciferous crops (particularly cabbage and oilseed rape) and several members of the families solanaceae, apiaceae and asteraceae (Parker & Riches, 1993).

O. foetida is widely distributed in natural habitats in the Western Mediterranean area (Portugal, Spain, Morocco, Algeria, Tunisia) parasitizing wild herbaceous leguminous plants of the genera *Anthyllis*, *Astragalus*, *Ebenus*, *Lotus*, *Medicago*, *Ononis*, *Scorpiurus* and *Trifolium* (Pujadas-Salvá, 1999; 2002). It is however considered an important agricultural parasite in faba bean in Beja region of Tunisia (Kharrat et al., 1992) with yield losses ranging 66-83% (Kharrat, 1999). This species seems to be more aggressive on faba bean and common vetch (*V. sativa*) than on other legumes (Kharrat, 2002). 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 *Lathyrus odoratus*, *L. sativus, Trifolium alexandrinum, Medicago truncatula* and *V. sativa* ssp. *amphicarpa* (Kharrat, 2002). It has recently also been found in Taounate, Morocco infecting common vetch (Rubiales et al., 2005b).

Other *Orobanche* species can infect leguminous plants, but they are generally of little economic importance. However, *O. minor* has a wide host range among forage legumes in temperate climates. It can cause important damage on clover growing for seed and has recently become a problem on red clover in Oregon, USA (Osterbauer & Rehms, 2002; Eizenberg et al., 2004b). It has been introduced into South and West Australia where it can be seen in gardens and in crop and pasture paddocks (Hussey et al., 1997) but is reported as a potential problem only for *Vicia ervilia* (Carter et al., 1996).

Other parasitic plants non belonging to *Orobanche* genera can also attack grain legumes and cause considerable yield reduction in semi-arid areas of sub-Saharan Africa like *Striga gesnerioides* and *Alectra vogelii* (Parker & Riches 1993; Rubiales et al., 2006). Being cowpea the most affected crop, yield losses range from 30-50% to the total crop loss (Aggarwal & Ouedraogo, 1989; Riches, 1989; Emerchebe et al., 1991).

- **Pathogenesis**

Before continuing, we would like to clarify a question concerning nomenclature of some structures of the parasite. The term 'haustorium' is commonly used in parasitic plants literature referring to both the attachment organ at the end of the parasite radicle and the endophyte (the structure connecting host and parasite tissues). We will reserve the term 'haustorium' for the endophyte, and following analogies with fungi, the term 'apresorium' will be used in reference to the attachment organ.

Orobanche spp. are holoparasites which attach to the root and connect to the vascular tissues of the host through a specialized structure known as haustorium. This structure allows an intimate contact between host and parasite, with the consequent exchange of water and nutrients (Press & Graves, 1995; Hibberd et al., 1999), hormones (Suzuki et al., 1994), toxins (Rank et al., 2004), and almost everything able to travel through vascular connections, including genes (Mower et al., 2004). But succeeding in the formation of such structure implies a complex process in which the parasite finishes its short independent cycle and began the parasitic life. The life cycle of root parasitic angiosperms like *Orobanche* or *Striga* is very similar. It comprises an independent phase and the proper parasitic phase (Joel, 2000). The fist phase is composed by several steps leading to the formation of the haustorium: preconditioning of seeds, germination, attachment and formation of the apresorium, penetration through host tissues, and formation of the haustorium and connection to the vascular tissues. During the second phase, the parasite growths at the expenses of the host, developing a small tubercle at the beginning, a crown of roots and an incipient apex. Later on the apex develops a stem which will emerge from the soil, will flower, and will set the seeds for a new cycle (Figure 1). The knowledge and understanding of each step involved in the process is crucial, because each step is potentially a target for the control of the parasite (Jorrín et al., 1999; Joel, 2000).

Many efforts have been devoted to fight parasitic plants on crops, but to date there are no completely satisfactory control measures. Limited success has been obtained using chemical control (Jurado-Expósito et al., 1996, 1997; Eizenberg et al., 2004a) and agronomical practices (Mesa-García & García-Torres, 1991; Manschadi et al., 1997), but none of them provides total control of the parasite. Genetic resistance remains as one of the most desirable components in an integrated control strategy. However, breeding for resistance to broomrape in legumes has been revealed as a difficult task: it has proven to be a polygenic character with very low heritability (Cubero, 1994; Rubiales, 2003). In addition, little resistance is available in commercial cultivars of crops like pea, so turning to wild relatives for breeding is required (Rubiales, 2003; Rubiales et al., 2003a; 2005a). For those reasons, understanding the mechanisms underlying host resistance is needed in order to improve the screening methods in breeding programmes.

MEDICAGO: MODEL FOR LEGUMES - PARASITIC PLANTS INTERACTIONS

The excellent traits of *Medicago truncatula* as a model plants for laboratory studies about legumes 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* amongst others (Fernández-Aparicio, unpublished results).

It is important to notice that despite most of the information we provide in this chapter is referred to studies of resistance, the model is also very important for develop studies about the pathogenesis of these parasitic plants in legumes. The knowledge of the mechanisms responsible for the pathogenesis of the parasite is crucial in order to develop strategies of control, including breeding for resistance.

Following these lines the reader will find three main sections: one dedicated to histological studies, another one explaining gene expression studies, and finally the last one centred on proteomic studies of the interaction between legumes and parasitic plants.

- **Histological studies**

Studying resistance against parasitic plants presents some drawbacks when compared with other pathosystems because parasite and host are relatively close organisms that share many morphological, physiological and biochemical traits. For example, bacteria and fungi release many specific enzymes that can be differentiated from plants enzymes. Furthermore, due to differences of cell wall constitution and morphology, bacteria colonies and fungi mycelia are easily distinguishable inside plant tissues. But when the interaction involves two plants which merge their tissues, its study is more complicated. For that reason, cytological and cytochemical techniques are powerful tools in order to unveil the mechanisms underlying the plant-parasitic plants interaction.

Preliminary studies

To date, the most numerous and important works about the histology of parasitic plants were centred mainly on the development in susceptible host, as it is the case of *Orobanche* spp. (Krenner, 1955; Musselman & Dickison, 1975; Dörr & Kollmann, 1976; Joel & Losner-Goshen, 1994; Neuman et al., 1999), *Striga* spp. (Dörr, 1997; Reiss & Bailey, 1998), *Cuscuta* spp. (Kuijt, 1977; Dawson et al., 1994; Vaughn, 2002; 2003), *Viscum* spp. (Heide-Jørgensen, 1987) and many others (Calvin, 1967; Kuijt, 1969; 1977; Musselman, 1975; Visser et al., 1984; Heide-Jørgensen & Kuijt, 1993; 1995). On the contrary, the basis of host resistance to these parasites is less known (Joel et al., 1996). Only in the last years some histological studies of the resistant interactions have been undertaken (Dörr et al., 1994; Antonova & Ter Borg, 1996; Goldwasser et al., 2000a; Labrousse et al., 2001; Rubiales et al., 2003b; Zehhar et al., 2003; Pérez-de-Luque et al., 2005c; 2006a, b). However, great gaps remain in our knowledge about this topic specially when compared

with studies of resistance against pathogenic fungi (Thordal-Christensen et al., 1997; Mellersh et al., 2002).

The usual phenomena observed and which characterise incompatible interactions described in the case of root parasitic plants are (Figure 2):

1. Stoppage of parasite seedlings penetration into the host root, usually (but not always) accompanied by darkening of host and/or parasite tissues around the point of attachment (Zaitoun et al., 1991; Dör et al., 1994; Lane & Bailey, 1992; Goldwasser et al., 1997; Serghini et al., 2001; Rubiales et al., 2003b; Pérez-de-Luque et al., 2005c; 2006b; Echevarría-Zomeño et al., 2006).

2. Darkening and/or death of established parasite tubercles (Dörr et al., 1994; Labrousse et al., 2001; Zehhar et al., 2003; Pérez-de-Luque et al., 2005a; 2006a).

Both defensive reactions can take place in either resistant or susceptible accessions, being the differences quantitative: a plant allows more or less parasite infection depending on the number of these defensive reactions found in the roots (Labrousse et al., 2001; Pérez-de-Luque et al., 2005a; Echevarría-Zomeño et al., 2006). However, the exact mechanisms of resistance governing these defensive reactions are not well known. For example, stoppage of seedling penetration has been usually associated to a hypersensitive response (HR), but there is no conclusive evidence that a HR really occurs in these interactions (Rubiales et al., 2003b; Pérez-de-Luque et al., 2005c) in a manner similar to that described for fungal attack (Heath, 1999; Richael & Gilchrist, 1999). For that reason, histological and histochemical studies are of great value in order to know what is really happening inside host tissues.

Something about the techniques

Several simple techniques are available to develop histochemical studies of the interaction between parasitic plants and legumes (see Inoculation and growth with root parasitic weeds, by Fernández-Aparico et al. in this Medicago Handbook). However, the main drawback is to get the roots of the host infected with the parasite and to follow the infection process in order to take samples at the right moment. In our working group we use a Petri dish system (Pérez-de-Luque et al., 2005a; Rubiales et al., 2006) for in vitro cultivation of the *M. truncatula* plants and inoculation with *Orobanche* spp. seeds (Figure 3). With this system is possible to characterise and quantify the defensive reactions and take the samples when is necessary, following the evolution of the interaction.

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 (Fernández-Aparico et al., 2007).

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). This, however, will be commented later.

The research nowadays

At present, we are developing histological studies of the interaction between *O. crenata* and *M. truncatula*. Resistant and susceptible accessions previously identified and tested in growing chambers under controlled conditions (Rodríguez-Conde et al., 2004) were selected. The two defensive reactions described above (stoppage of parasite seedlings penetration and death of established parasite tubercles) have been detected and used for histochemical analysis (Pérez-de-Luque et al., 2005b).

Preliminary results have shown that the penetration of *O. crenata* in resistant *M. truncatula* is stopped once the parasite intrusive cells have reached the host central cylinder (Figure 4A). This differs with what have been detected in legumes resistant to *O. crenata*: the parasite intrusive cells are stopped in the host cortex, either before (Pérez-de-Luque et al., 2005c) or after (Pérez-de-Luque et al., 2006a) reaching the endodermis, but the parasite never penetrates the central cylinder of the resistant host as it happens in this particular case. So the stoppage of parasite seedlings penetration into the host root seems to be associated to another mechanism of resistance different of that described to date for other resistant legumes (Pérez-de-Luque et al., 2005c; 2006a).

Staining of samples with some of the procedures described above showed accumulation of substances in the apoplast within and around the penetration pathway of the parasite, and the parasite tissues presented a disrupted and disorganised aspect. Host cells in contact and near the parasite tissues were impregnated with these substances, which also reached and filled the host xylem vessels in some cases. A strong blue-white fluorescence was detected from the walls and middle lamellae of cells surrounding the parasite intrusive tissues, including some xylem vessels (Figure 4B). A wall thickening inside host xylem vessels in contact with parasite tissues was also observed in incompatible interactions.

All these data suggest that some defensive mechanisms are set in motion. Firstly, a thickening of xylem walls in contact with parasite cells takes place, probably by accumulation of lignins and/or polyphenols. This would prevent the parasite cells from establish connections with host vessels as has been previously described for other host-*Orobanche* interactions (Dörr et al., 1994). Secondly, accumulation and secretion of toxic metabolites like phenolic compounds at the infection zone finally will kill the parasite, as suggested by the fluorescence observed in host cells in contact with parasite cells (Figure 1B). Accumulation and secretion of phenolic compounds against broomrape has been also reported in the case of *O. cumana* and sunflower (Serghini et al., 2001; Echevarría-Zomeño et al., 2006).

On the other side, the first histological studies of darkened established tubercles with AGS have shown a dark substance accumulating in the apoplast and host vessels connected with the parasite vessels (Figure 4C). This dark deposit does not stain with AGS and quenches the wall fluorescence of the affected cells and vessels. No presence of mucilage inside host vessels has been detected, as previously described for *O. crenata-Vicia sativa* incompatible interactions (Pérez-de-Luque et al., 2005c; 2006b). For that reason, the most feasible explanation is that the host is poisoning the established parasite through vessels connections secreting some kind of toxic substance, but more studies are needed in this way.

- **Gene expression studies**

Although some genomic studies have been aimed to identify and locate *Orobanche* resistance genes /QTLs in molecular maps in legumes (Román et al., 2002; Valderrama et al., 2004), the host response to parasitic plants seems to require a more deep understanding of the complex multifactorial process aimed to defend itself even as its metabolism is redirected to feed the attached parasite. In this sense, although some molecular markers have been proposed to assist breeders by means of MAS against *O. cumana* (Lu et al., 2000; Tang et al., 2003), there is a strong consensus that detailed knowledge of the molecular mechanisms underlying the host-parasite interaction is necessary to improve breeding programs. Nevertheless, little is known so far about the changes of gene expression in parasitized plants and the molecular basis of root-parasitic plant association, probably due in part to the scarcity of resistant germplasm, in part to the complexity response of the host plant. Compared with the number of histological studies described above or with the intensive research carried out into symbiotic associations involving plants, studies on parasitic angiosperms and their interaction with host plants (particularly, legumes) at the molecular level are very limited. In recent years, high-throughput expression profiling technologies have transformed significantly molecular genetics approaches in legumes. The emergence of the "omic" technologies and the establishment of *Medicago truncatula* as a model legume plant are promising strategies for understanding the molecular genetic basis of stress resistance, including parasitic plants.

Single-gene expression studies

Most of the gene expression analysis performed in host-plant-parasite interaction has consisted on the study of single or few genes. Generally *Orobanche* spp. have been used as the parasite since many economically important hosts, e.g. sunflower, tomato, tobacco and legumes are severely affected by this genus. Taking advantage of their ability to form pathogenic association with model organisms such as *Arabidopsis thaliana* or *Medicago truncatula*, these model plants has been also used for this purpose (Westwood & Foy, 1998; Goldwasser et al., 2000b; Rodríguez-Conde et al., 2004).

Although little is known about the biosynthesis pathways involved in defence mechanisms or how these pathways are regulated, some studies regarding the dissection of the molecular response in the host have been carried out. In this sense, the induction of a PR-1 gene promoter from tobacco in roots infected with *O. aegyptiaca* has been reported at

the infection site (Joel & Portnoy, 1998). The expression of the PR gene promoter in the host roots indicates that *Orobanche* releases appropriate elicitors, and that the transgenic tobacco plants do sense *Orobanche* invasion, in spite of the fact that they do not resist haustorial invasion. Moreover, Westwood et al. (1998) demonstrated that parasitization by *Orobanche* induced the expression of *hmg2*, a defence-related isogene of 3-hydroxy-3methylglutaryl CoA reductase (HGMR) in transgenic tobacco as early as 1 day after root penetration. This protein is involved in isopropenoid biosynthesis pathway and is activated specifically during defence responses associated with phytoalexins and sesquiterpenes production. The enhanced expression was spread in the cortical and vascular tissues of the host, around the penetration site, and persisted on infested roots for 4 weeks. The demonstrated induction of a pathogenesis-related protein PR1 gene in response to *Orobanche* attack (Joel & Portnoy, 1998) together with the activation of a *hmg2* promoter (Westwood et al., 1998), indicates that *Orobanche* parasitization may trigger a broad array of defence responses that are common to pathogen attack such increased production of phytoalexins, lignin precursors, and lyctic enzymes (Lamb et al., 1989).

In addition to phytoalexins, the isoprenoid pathway produces many important compounds needed for general metabolism and growth. A second isogene encoding HMGR, *hmg1*, is expressed in processes related to cell division and growth (Denbow et al., 1995). Another enzyme, squalene synthase occurs downstream the pathway and seems to be coordinately regulated with *hmg1* to divert carbon toward the production of sterols (Chappel, 1995). The expression patterns of *hmg2, hmg1* and squalene synthase has been analyzed in tomato roots parasitized with *O. aegyptiaca* (Griffitts et al., 2001) indicating a coordination of metabolic events in the isoprenoid pathway. In this study, the host plant appears to recognize the mechanical wound that occurs during parasite attachment, but also perceives *O. aegyptiaca* as an invasive pathogen, promoting the alteration of gene expression in the host plant by activating *hmg2* and repressing the squalene synthase involved in the general metabolism, thus mobilizing the host energy toward the production of defence compounds. Recent *in situ* hybridization studies have also shown that a peroxidase and a β-glucanase are differentially expressed in cells of pea roots resistant to *O. crenata* (Pérez de Luque et al., 2006a). In both cases the expression was restricted to cortical cells and only to those near the parasite intrusive cells.

An accurate kinetic study of the gene expression pattern in host roots from the earliest contact with *Orobanche* seed to the installation of a functional haustoria, could also contribute to better understand the mechanisms involved in the host defence against the parasite. In this sense, Dos Santos et al. (2003a) studied the effect of *O. ramosa* on the expression patterns of *Arabidopsis thaliana* genes that were known to be involved in metabolic pathways and resistance mechanisms along different time points. Several defence genes that are involved in the jasmonate and ethylene signalling pathways were upregulated in response to germinating *O. ramosa* seeds. This response was detected even before the attachment of the parasite indicating that signalling from the *Orobanche*-germinated seed is recognized by the host plant that, although susceptible, is able to activate several defence reactions against its aggressor.

Transcriptional profiling analysis: understanding of biological process

Analysis of gene expression has moved rapidly from classical studies on single or few genes toward genome-wide studies on multiple genes providing a more integrated view of biological processes. In relation to more than a thousand genes identified as up-regulated during different steps of root nodule initiation and function during symbiotic relationship with rhizobial prokaryotes or activated during arbuscular mycorrhiza, the knowledge of *Orobanche* induced genes in legume plants is still very limited. As far as we are concerned, the only molecular study published with *O. crenata*-legume system was a proteomic approach comparing resistant and susceptible pea cultivars (Castillejo et al., 2004).

In the genomics era, a more comprehensive view of gene induction during the infection process should be possible, and experiments making use of suppression subtractive hybridisation cDNA libraries (SSH), cDNA-array hybridizations, and real-time reverse transcription (RT)-PCR experiments could identify *M. truncatula* genes regulated during the plant-parasite interaction. In particular, the fact that *M. truncatula* can be infected by *O. crenata* (Rodríguez-Conde et al., 2004), has opened the possibility of using this model plant to gain knowledge regarding parasitic plant-legumes interaction

Microarray studies allow a more comprehensive understanding of molecular responses in the infection process and the elucidation of possible mechanisms involved in resistance. Thus, our group started to monitor genes induced in *M. truncatula* in response to *O. crenata* infection using the recently developed M16kOLI1 microarray that screens the expression of more than 16,000 genes in the frame of the "Grain Legumes" European Union FP6 Integrated Project. For this, expression profiles of two genotypes of *M. truncatula* with different resistance mechanisms (complete resistance acting at early penetration stages and incomplete late acting resistance mediated by necrosis of parasite tubercle), were obtained and compared at three infection stages: radicle contact, tubercle initiation and mature tubercle formation. This approach allowed the identification of significant changes in the steady-state levels of many transcripts belonging to several functional categories, including common pathogen-induced genes, such as PR genes, hormone-associated genes and transcription factors. Interestingly some of these genes as those encoding to patatin, lypoxigenases and glucanases were also up-regulated in the *Arabidopsis-O. ramosa* interaction. Data analyses also revealed the activation of both the salicylic acid and jasmonate defence-pathways (Dita, unpublished results).

Nevertheless, most genes present on this microarray are from symbiotic association random cDNA libraries and additionally, due to the tight shown regulation of significant plant genes with respect to the timing and the localization of expression, they tend to be underrepresented in random cDNA libraries, and hence, are difficult to detect on the basis of microarray hybridizations. Thus, other strategies are necessary in order to identify new and/or specific differentially expressed genes in the *M. truncatula-O. crenata* interaction. SSH enables the construction of cDNA libraries enriched for genes up-regulated under certain conditions (Diatchenko et al., 1996). This transcriptomic approach has become very effective to obtain global views on gene regulations changes during different plant-pathogen or microbe interactions in general (Nyamsuren et al., 2003; Frenzel et al., 2005) or *A. thaliana-O. ramosa* in particular (Dos Santos et al., 2003b). Taking this in mind, we

constructed a cDNA-SSH library in order to identify *M. truncatula* genes differentially expressed during infection by *O. crenata* (Die, unpublished results) As far as we aware know, our ESTs collection represents the first application of SSH to study *Orobanche*-induced genes in *M. truncatula*. Sequencing, clustering and annotation of ESTs obtained, resulted in most gene functions already known to be involved in different plant responses pathways and resistance mechanisms activated during several plant-pathogen interactions. Nevertheless, a notable numbers of ESTs (11.78% of sequences in the library) were derived from novel genes not matching entries of large-scale *M. trucatula* sequence collection indicating that these gene products could be good candidates to play rather specific role during the infection process. Data presented in our study with *M. truncatula* could be used in future projects aimed to the characterization of gene function and regulation involved in defence reactions of economically important grain legume crops.

Future perspectives

Undoubtedly, the model system *M. truncatula-Orobanche* spp. will continue to play a critical role in contributing to our understanding of the mechanisms underlying legume defence response. A major challenge for comparative legume genomics is to translate information gained from model species into improvements in crop legumes. *M. truncatula* has provided an impressive array of genomic tools to legume community and its direct application should make plant breeding programs easier and more efficient. Additionally, as genes differentially expressed on the parasite during infection process may have a decisive role in the modulation of the host defence reactions. Simultaneous analysis of genes from the two partners of the interaction should increase our understanding of the successive attack and defence steps leading to plant resistance or susceptibility. The use of a single microarray to simultaneously probe gene expression in two interacting organisms (interactome) as reported in the pathosystem *Phytophtora sojae*/ soybean (Moy et al., 2004), could be also exploited in the future to study the interaction *Medicago truncatula-Orobanche spp.* This will be particularly possible once the genomic and expression studies in *Orobanche* spp increase, allowing the access to large databases of the parasite in the near future. The fact that *O. crenata* is also a higher plant makes this pathosystem an exciting, intriguing and fascinating target of research.

Finally, the combination of genetic and transcriptomic approaches (mapping and arraying) could be also proposed to gain substantial information in the understanding of plant resistance by finding the relationship existing between the loci identified and located in a molecular map and the transcription of genes involved in defence mechanisms. The use of oligonucleotides chips with labelled total genomic segregant DNA rather than mRNA should be a way to detect single feature polymorphism and could provide a link between a given phenotype and the genes putatively involved in its resistance. Advances in our understanding of the multifactorial complex response to parasitic plant will be achieved thanks to both, basic and applied genomic of this model legume plant.

- **Proteomic studies**

Why Proteomics?

The beginning of the 21st century marks the "post-genomic era", era of functional genomics, which goal is understanding gene expression and function at a large scale. Proteomics, considered either as a scientific area or a methodological approach, deals with the study and characterization of the cellular proteome, defined as the set of protein species present in a biological unit (organism, organ, tissue, cell or organelle), at a specific developmental stage and under determined external biotic and abiotic conditions. The advances in proteomics is a direct result of genomics and have been made possible due to improvements in protein separation either by two dimensional-gel electrophoresis (2-DE) (Görg et al., 2000), staining and scanning technology, or by multidimensional liquid chromatography (MudPIT) (Washburn et al., 2002), peptide sequencing by mass spectrometry (MS) (Steen & Mann, 2004; Venable et al., 2004), and bioinformatics (Apweiler et al., 2004; Liska & Shevchenko, 2003). Proteomics, considered as protein biochemistry on an unprecedented, high-throughput scale, is definitely required in order to get further insight into the molecular basis of any life process.

Proteomic studies are justified for several reasons. First, proteins are the effectors of biological functions, second, there is a high number of genes that codifies for proteins with no assigned function, third, the information obtained by using a transcriptomic approach is not complete (considering, i.e., post-translational modifications, and protein-protein interactions), and finally, in many cases the correlation between mRNA and protein levels is remarkably and unexpectedly low (Gygi et al., 1999; Jansen & Nap, 2002; Watson et al., 2003).

Proteomics is expected to revolutionise plant research providing new opportunities to deepen our knowledge in plant biology and crop improvement. However, its application to plants is still rather limited as compared to other biological systems. Even though proteomic research in plants has not advanced at the same pace as have human and yeast proteomics, this field has undergone exponential growth over the last few years showing as yet no sign of tapering off. The increasing activity within the area of Plant Proteomics can be estimated by looking at the publication output: since the period 1999-2003, (reviewed in Cánovas et al., 2004), 200 original papers have appeared describing different aspects of plant biology (reviewed in Rossignol et al., 2006).

Technological platforms: problems and limitations

From a methodological point of view, different strategies can be used for protein analysis. The first one uses classical biochemical techniques for protein separation (electrophoresis and/or chromatography) coupled to MS for analysing individual proteins, the identification of which being obtained following peptide mass fingerprinting (PMF) or *de novo* sequencing by using specific algorithms to search within protein, genomic or ESTs sequencing databases. This is called "classical proteomic approach". The second would implicate the generation of a set of clones that express a representative of each protein of a

proteome followed by the analysis on a genome-wide basis (genomic wide approaches) (Phizicky et al., 2003).

The classical approach, including electrophoretic separation and MS analysis, has been until very recently almost the only approach used in plant studies. New platforms such as multidimensional protein identification technology (MudPIT) have occasionally been used with very promising results especially with hydrophobic proteins (Koller et al., 2002). As deduced from a number of studies in which the number of proteins analysed and identified by electrophoresis or chromatography have been compared, none of the two separation methods (gel and non-gel based separation) can be considered better than the other, but both provides complementary information.

Concerning methodological aspects, sample preparation is the first and most critical step for subsequent protein separation and identification. The procedure used must allows extraction and solubilization of the maximum number of proteins avoiding proteolytic degradation. As a consequence of the intrinsic protein heterogeneity, the huge number of protein species, much higher than the number of genes, and the differences in abundance between proteins, there is not a unique suitable protocol for extracting and solubilising all proteins present in a given sample. Instead the plant researcher must select the protocol that better adjust to the experimental system and to the purpose of the experiment in each case. Protein extraction protocols for whole proteomic analysis have proliferated, with specialisation to plant organ (leaf, roof, cell suspension), and with TCA-acetone precipitation and phenol extraction proving to be most generically useful (Carpentier et al., 2005). While the latter allows for the simultaneous analysis of proteins and mRNAs (Dumas-Gaudot et al., 2004), metabolites, proteins and RNA have been sequentially extracted using a different protocol (Weckwerth et al., 2004) Precipitation protocols have been optimized to cope with small starting amounts of tissue, which is essential given that source tissue is often limited, and plant organs are composed of different cell types, each having its own proteome signature (Majeran et al., 2005). This is particularly relevant for the description of the response to pathogens and parasitic plants, as only a restricted number of cells play any active role in defence. In addition, the study of low copy number species and proteins with extreme values of pI and Mr are still a problematic task.

Another critical parameter is the election of the staining method for visualizing and analyse proteins from 2-DE gels. Several dyes are available, being the most commonly used coomassie, silver and fluorescent dyes differing in sensitivity and dynamic range. Besides, differences in the protein pattern of a given extract has been observed when using each staining method (Jorrín, personal communication). The method of election must be compatible with the consequent analysis by MS. Traditionally, coomassie staining was used, but recently is being substituted by some fluorescent dyes, such as SYPRO, equivalent to silver staining in terms of sensitivity but showing a broader linear range. (Chevalier et al., 2004).

Additional challenges for the plant research community flow from the fact that only experiments conducted on species which genomes are sequenced to completion can take full advantage of the technological cutting edge, as a fully sequenced genome is needed for the positive identification of proteins from MS data. It is clear that plant proteomics will

progress in pace with genomic progress, and the expectation is that the coming years will see an increasing number of fully sequenced and annotated plant genomes.

The state of the art

Even though plant proteomics studies have not advanced as the same pace than yeast and human proteomics, this field has experimented an exponential growth, with a figure close to 200 original papers appeared in the period 2004-2006 (Cánovas et al., 2004). These works have described aspects of the proteome of at least 35 plant species. Most focus either on the model species *Arabidopsis thaliana* and rice (*Oryza sativa*). The complete genome sequence of both these species is in the public domain, and this simplifies the identification of proteins from mass spectrometry (MS) data. Second to the two major model species are the major cereal, legume and solanaceous crops, and the model legume *Medicago truncatula.* All of these species are associated with significant quantities of published genomic DNA and EST sequence.

Plant proteomics studies cover different aspects of both basic and applied plant biology research, the scientific objectives ranging from a global description of proteomes and subproteomes (organ, tissue, subcellular compartments) to the study of biological physiological or biochemical processes (developmental stages, responses to environmental biotic an abiotic stresses, signal transduction pathways, post-translational modifications (PTMs), genotype characterisation and phylogenetic studies. In plant interactions with other organisms and plant defence responses to biotic stresses, few studies has been carried out that have focussed on signalling processes. All these aspects have been extensively reviewed and summarized (Rossignol et al., 2006; Agrawal & Rakwal, 2006; Jorrín et al; 2006; James et al., 2006; van Benten et al., 2006; Aro et al., 2005; Thurston et al 2006; Cánovas et al., 2004; Peck et al., 2005; Baginski et al., 2005).

The study of economically important legume crops such as soybean, pea, alfalfa, bean or chickpea is complicated because of their large genome sizes or polyploidy. Fortunately, due to their suitability for plant genomics and with the aim of getting insight into agronomic important legume-microbe interactions, *Medicago truncatula* have emerged during the last decade as model systems for legumes (www.medicago.org; Handberg and Stougaard, 1992). This is a convenient model for proteomic studies, as their genome sequencing is under way and large collections of ESTs databases corresponding to various biological situations are available on line. Most legume proteomic papers published up to 2004, covered by the review by Jorrín et al. (2006), dealt with the model system *Medicago truncatula*. A few proteomics studies have successfully been conducted with other legume species, mainly soybean (David et al., 2004; Maruyama et al., 2003; Mooney and Thelen, 2004), and to a lesser extent pea (Fecht-Christoffers et al., 2003; Repetto et al., 2003; Schiltz et al., 2004) and alfalfa (Watson et al., 2004). In the last two years (reviewed in Rossignol et al., 2006) several original papers appeared on pea dealing with pathogen/parasitic plants –plant interaction (Curto et al., 2006; Castillejo et al., 2004) and soybean concerning studies on seed proteome, and analysis of symbiotic nodulating roots (Natarajan et al., 2006; Hollung et al.,. 2005; Sarma y Emerich, 2005; Wan et al, 2005)..

Projects directed to study the *Medicago truncatula* proteome have been initiated in different labs worldwide and 2-DE protein map databases are available on line (http://www.noble.org/2dpage/search.asp; http://semele.anu.edu.au/2d/2d.html), concerning different aspects such as protein profiling of specific tissues and cell cultures (Watson et al., 2004; http://www.noble.org/PlantBio/MS/proteomics.html), proteome reference maps of roots and embryogenic cell cultures (Mathesius et al. 2001; Imin et al. 2004; http://semele.anu.edu.au/2d/2d.html) and the proteome of nitrogen fixing-legume symbioses (Rolfe et al., 2003; Mathesius et al. 2003a,b).

Compared to the number of proteomic publications in the field of beneficial plant-microbe interactions (nitrogen-fixing symbioses and arbuscular mycorrhizal symbioses), the use of proteomics appears rather limited in the area of plant responses to pathogens (reviewed in Jorrin et al., 2006). Mathesius et al., (2003b) has described the *M. truncatula* responses to the pathogenic bacteria *Pseudomonas aeruginosa* and Colditz et al., (2004) analyzed the root protein profiles of *M. truncatula* after *Aphanomyces euteiches* pathogen infection. Following MALDI-TOF-MS analyses and PMF, a number of spots that were differentially expressed in response to the infection were identified. Recently, Curto et al. (2006) studied the responses to the fungal pathogen *Erysiphe pisi* of two pea genotypes (one resistant, the other susceptible), identifying several putative proteins involved in constitutive and induced resistance.

The group of Jorrín at the University of Córdoba (Spain) has initiated a project in which plant responses to biotic and abiotic stresses are studied using a proteomic approach in *Arabidopsis*, model and crop legumes, and sunflower. Within the EU Grain Legumes Project, resistance to pathogens (*Uromyces, Erysiphe* and *Mycosphaerella*) and parasitic plants (*Orobanche crenata)* in *M. truncatula* and *P. sativum* are being studied at the proteomic level (Curto et al., 2006; Castillejo et al., 2004). Concerning parasitic plants, the group of Jorrín was pioneering in applying Proteomics, as a global non-targeted approach on parasitic plant research using three different experimental systems: *P. sativum-O. crenata* (Castillejo *et al.*, 2004), *M. truncatula-O. crenata* (Castillejo, personal communication) and sunflower-*O. cumana* (Echevarría, 2005).

The general strategy utilized for these experiments comprises 2-DE protein profile comparison of resistant and susceptible genotypes, spots showing quantitative and qualitative differences in the protein profile between genotypes are extracted from the gel, purified, digested and analysed by MS and identification by DNA, EST or protein database searching using specific algorithms (i.e. MASCOT) (Figure 5). By comparing the proteins present in root extracts from genotypes differing in resistance, in control and infected plants, a global knowledge was gained on the molecular events mediating pea (*Pisum sativum*) and *Medicago-Orobanche crenata* interaction and these supporting resistance (Castillejo et al., 2004; personal communication).

First a comparative proteomics analysis was conducted on *Pisum sativum* using two pea accessions displaying the most contrasted behaviour against *O. crenata*. (Castillejo et al., 2004). Spots differentially expressed were analysed by MS and some of them identified by database searching. Some clear tendencies can be stated from these studies. As it has been observed for other experimental systems (i.e. Curto et al., 2006), most of the differential

proteins identified appear to be either constitutively present (pre-formed defences), or are specifically induced in the resistant/tolerant plants. For some differential proteins, multiple isoforms or specific PTMs could be detected. The majority of the differential proteins present in resistant and susceptible genotypes (as a general rule, in increased amount in resistant types, and in decreased amount in susceptible ones) belongs to two major categories: i) defence- or stress-related, and ii) enzymes associated with C and N metabolism, and mitochondrial electronic chain. Within the first group are the PRs (such as glucanases, chitinases, proteases), peroxidases and ABA-responsive proteins. Irrespective of *O. crenata* infection the higher constitutive presence of these proteins in the resistant genotype could help the plant in priming defence reactions more rapidly upon attack. Upon pathogen attack, some of these proteins are further induced, contributing very likely to host defence responses. The role of peroxidases and glucanases in legume responses to parasitic plants is well established by transcriptomic and histological approaches and has been commented in previous chapters. Within the second group, a number of enzymes associated either with carbohydrate assimilation and metabolism (including fructokinase and fructose 1,6-bisphosphate aldolase), nitrogen assimilation (gluthamine synthetase) and metabolism (ferredoxin NADP+ oxidoreductase) have been identified. This is not surprising as plant carbohydrate and N metabolism plays an important role in plant-parasitic plants interactions. The observed metabolic changes can be reflecting either a decrease in the photosynthetic activity occurring in infected plants, or a decrease in the availability of the translocated sucrose to the host cells as a consequence of the sink potential of the installed broomrape. The fact that two metabolic pathways (carbon and nitrogen) seemed to be affected in relation to susceptibility/resistance of the pea genotypes has opened up new possibilities to better understand the re-direction of host assimilates from host sinks to the parasite. In addition, these data strongly support the existence of similar defence strategies against a broad range of pathogens - including bacteria, fungi, and parasitic plants.

The results obtained from the pea system validates proteomic as a holistic approach to study legumes-broomrape interactions. Nevertheless, a complete success into the protein identifications would have improved the interpretation of the behaviour of both genotypes against broomrape infection. Consequently, the same group has conducted a similar study using *Medicago*, a more tractable biological system that would facilitate molecular, genetic, and mutational approaches to analysing the parasite-legume interaction, and get insights into the molecular bases of legumes- *Orobanche crenata* interaction and resistance.

Using the same general approach mentioned before, the root proteome of two genotypes, displaying different degrees of resistance to the parasite (resistant SA 27774 and moderately resistant SA 4087) in control and infected plants were compared.

The protein profiles of *M. truncatula* root tissues from the different situations compared (genotypes, inoculation, sampling times) were established after 2-DE: on 3-10 pH gradient, IEF strips as the first dimension, and 12% polyacrylamide home-made SDS-PAGE, as the second. After silver and coomassie staining approximately 600 and 300 well-resolved spots were detected respectively (Figure 5) representing only a minimal fraction of the root proteome. Nevertheless it was sufficient to detect both qualitative and quantitative differences between the different conditions analysed using PD-Quest^{TM} software (136 in total). 69 spots showed differences between non-infected, SA 4087 and SA 27774 plants,

42 spots between non-infected and infected SA 4087 plants, and 25 spots between non-infected and infected SA 27774 plants.

Protein spots with differential accumulation patterns were then identified by coupling a mass spectrometry analysis on a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) system with data mining of Expressed Sequence Tag (EST) and protein databases. This allowed identification of 50 differential spots.

Among the spots differentiating genotypes were proteins related to stress and defence responses, being some of them further modified after parasite attempt of infection. These results are in agreement with previous results obtained in pea, being some of the identified proteins common to both experimental systems. Additional defence and stress-related proteins were identified in *Medicago*, such as glutathione-S-transferase, several trypsin inhibitors and glycine –rich RNA binding protein. The differences observed between both genotypes could account for different types of resistance. For example a chalcone-flavone isomerase, was only detected in roots of one of SA 4078 genotype. It is the second enzyme of the flavonoid-isoflavonoid pathways in legumes leading to phytoalexin production, which induction and accumulation is a typical defence response associated with resistance in several plant systems and against a diverse range of attacking organisms including parasites (Shergini et al., 2001). These results further validate the use of *Medicago* as a model system to study plant-parasitic plant interactions allowing the identification of proteins very likely involved in resistance to *Orobanche* and in protecting the host plants against oxidative stress generated as a consequence of parasite attempt of penetration.

Future prospects

While the state-of-the art technology for proteomic analysis is to hand, the major obstacle preventing extension of this field beyond the model species is the fact that only experiments conducted on fully sequences species can take full advantage of the current technology. It is clear that plant proteomics will progress in harness with genomic progress, and the expectation is that the coming years will see an increasing number of fully sequenced and annotated plant genomes. The huge amount of empirical information derived from proteomics studies should, in turn, assist in generating plant-specific databases (organelle-, tissue-, stress response-specific) and efficient and accurate bioinformatics prediction programs to infer localization, PTMs and functional and interacting domains.

An important challenge concerns the development of methodology, in relation to the establishment of easy, reliable and efficient protocols for sample preparation, reducing the amount of starting material which is essential given that source tissue is often limited. This is particularly relevant for the description of the response to pathogen attack and parasitic plants, as only a restricted number of cells play any active role in defence, resulting in the proteins involved in such response being considerably diluted in the total protein extract. In this sense laser micro-dissection allows for the collection of homogeneous tissue- and cell-specific plant samples and might help to solve the problem of protein dilution (Schad et al., 2005).

Another challenge concerns the objectives of legume proteomics studies. Once the completion of *Medicago* protein directories is completed, new plant proteomics studies will be more likely directed towards functional analysis. Hence, protein quantification will grow in importance. For such purpose the standardization of protocols for tissue fractionation, protein extraction and solubilization will accelerate the use of leading edge proteomic technologies that has been already successfully used in mammals and yeast. These include non-gel based separation techniques such as liquid chromatography (LC), including MudPIT, both gel-based (such as DIGE [difference gel electrophoresis]), and LC-based (such as ICAT [isotope coded affinity tags]) techniques, stable isotope labelling by amino acids in cell culture (SILAC), cleavable stable ICAT, and the modified version iTRAQ (isobaric tags for relative and absolute quantitation) is a means towards comparative proteome analysis (reviewed in Rossignol et al., 2006). These technology has been already validated for plant studies in *Arabidopsis* and will be very useful to measure relative protein abundance by MS The use of these technologies in combination with pre-fractionation, and enrichment protocols for specific subgroups of proteins will enable quantitative studies with an unprecedented level of sensitivity, covering entire subproteomes and the investigation of their dynamic behaviour throughout specific processes such as responses against parasitic plants.

The ultimate goal of proteomic technology is to define protein function. Although high-throughput experiments typically generate multiple candidates as components of a biological response, the critical demonstration as to which of these do indeed participate in the process under investigation is needed. In summary, the combination of proteomic technology with genomics, genetics, plant pathology, histology and physiology is expected to increase our knowledge on protein functions in legumes, including responses to parasitic plants.

Progress in these directions will lead to the modelling of entire metabolic pathways in the coming years and thus usher in an era of predictive biology. This will represent a giant step for biotechnology, allowing it to contribute significantly to the design of genetic solutions to the ever-present threats of biotic stresses including infection with parasitic plants.

CONCLUDING REMARKS

Studies about the interaction between parasitic plants and their host are yet under preliminary development. However, thanks to model plants like *Arabidopsis* and *Medicago* it will be possible to gain a rapid and strong knowledge about the interaction during the next years. The development of new techniques and their transference from other fields of research will increase and it is possible that in a few years we will learn more about this pathosystem than in the past fifty years.

The research in the fields of gene expression and proteomic will allow the identification of numerous genes implicated in the processes of pathogenesis and resistance to parasitic plants. These techniques allow mass studies of the genes in question. But in order to validate those results and elucidate the pathways implicated in resistance and pathogenesis, more detailed studies with selected genes will be required. For that reason, molecular

histology is needed to locate accurately the expression of the genes in plant tissues, and immunocytology will help to locate the proteins in the same way.

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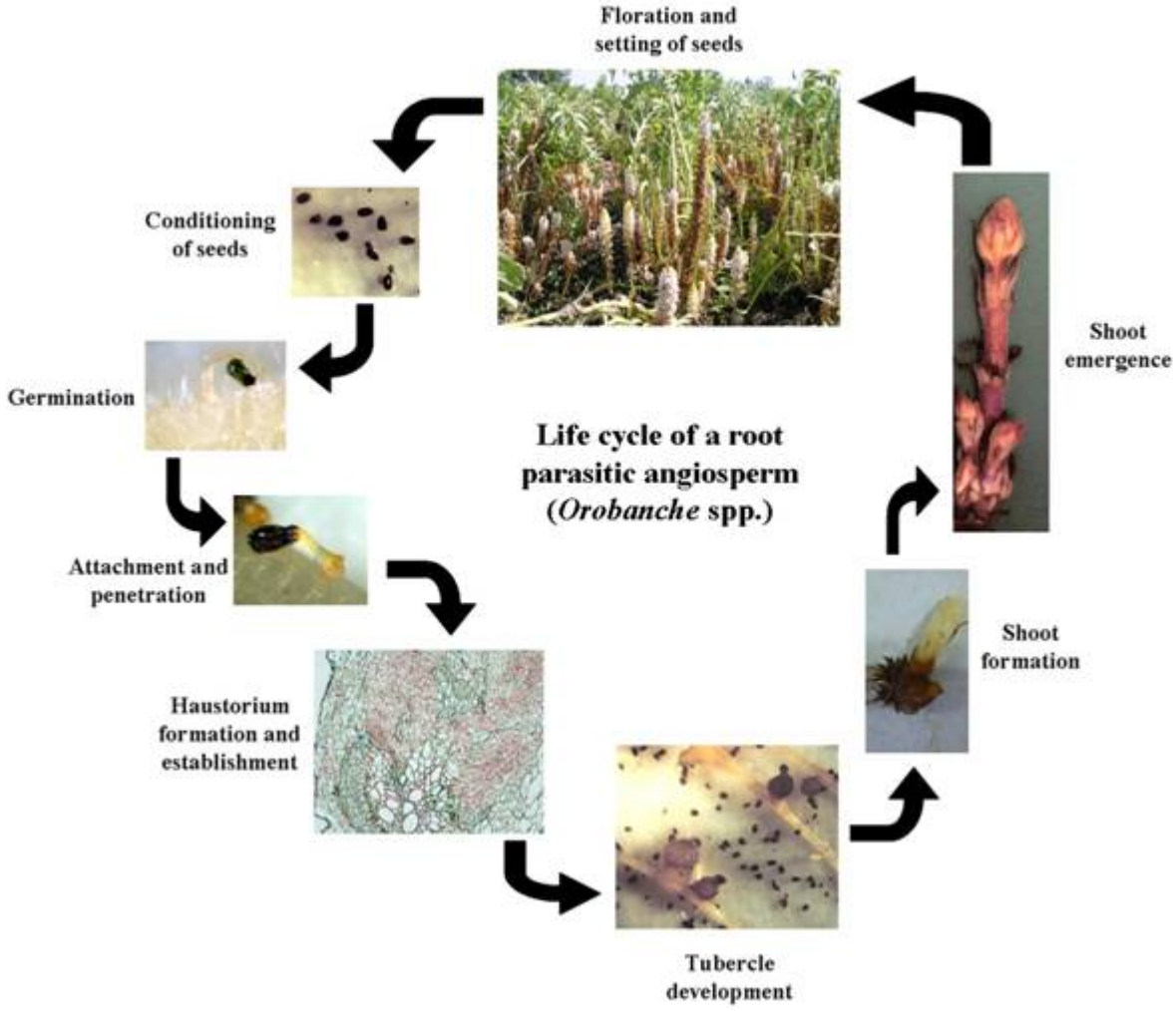

Figure 1: Schematic representation of the life cycle of a root parasitic angiosperm.

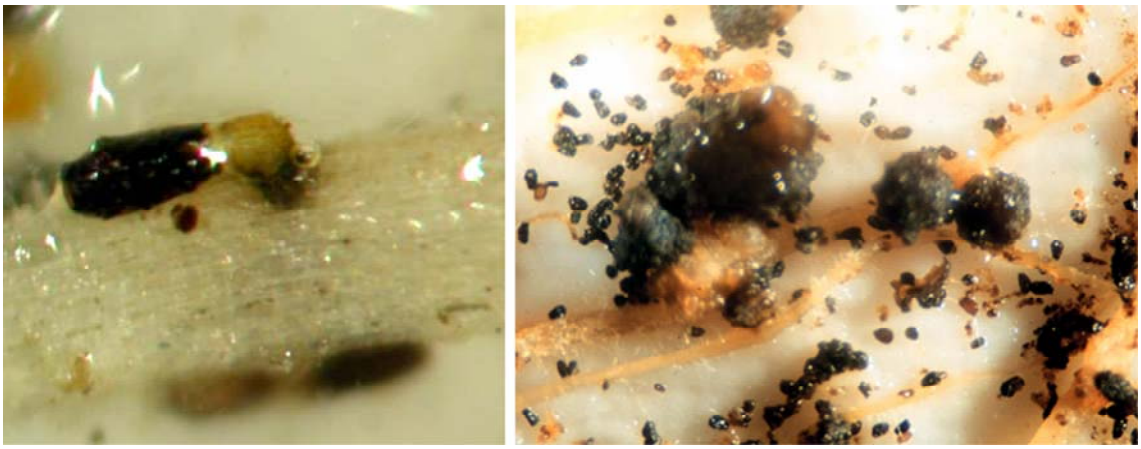

Figure 2: (A) *Orobanche* seedling unable to penetrate into the host root. A dark area surrounds the penetration point. (B) Darkened *Orobanche* tubercles on resistant host.

Figure 3: Pea infected with *Orobanche crenata* using the Petri dish assay.

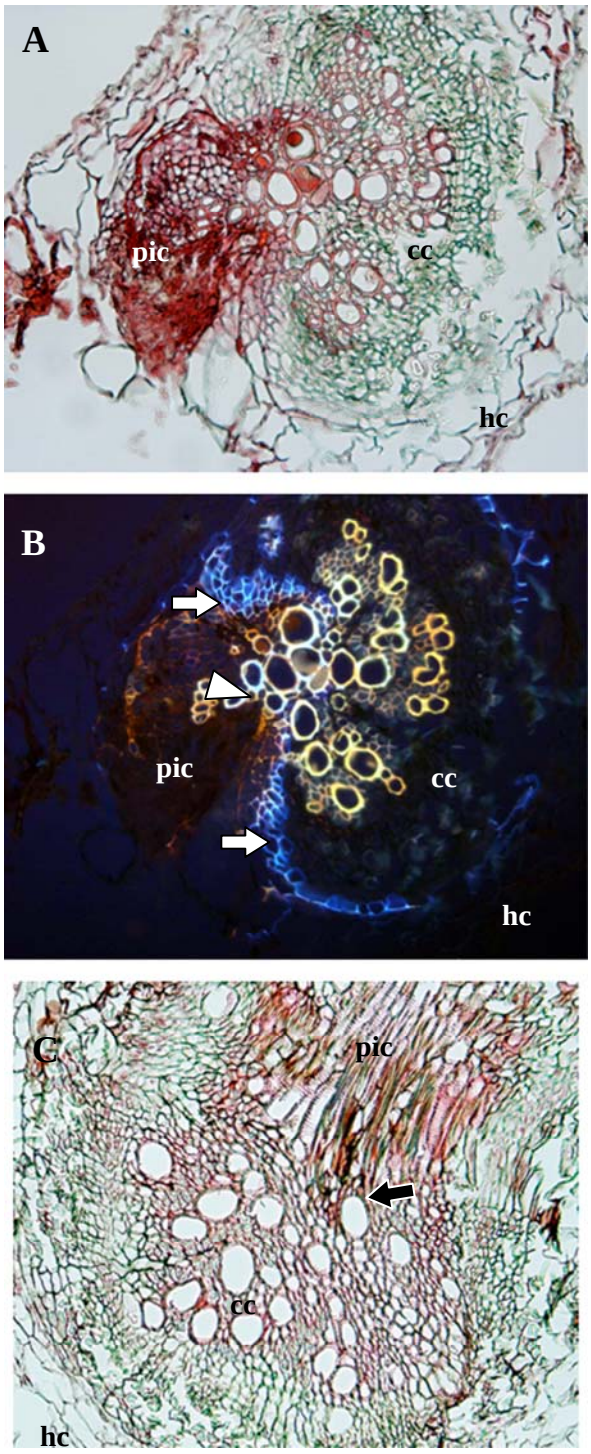

Figure 4: (A) Cross section of an unsuccessful *Orobanche crenata* seedling penetration on resistant *Medicago truncatula*, stained with AGS. (B) idem as A observed under UV exitation showing fluorescence from the walls and middle lamellae of cells surrounding the parasite intrusive tissues (arrows) and xylem vessels (arrowhead). (C) Cross section of a dark *O. crenata* tubercle on *M. truncatula*, stained with AGS, showing accumulation of a dark substance in parasite vessels (arrow). pic, parasite intrusive cells; hc, host cortex; cc, host central cylinder.

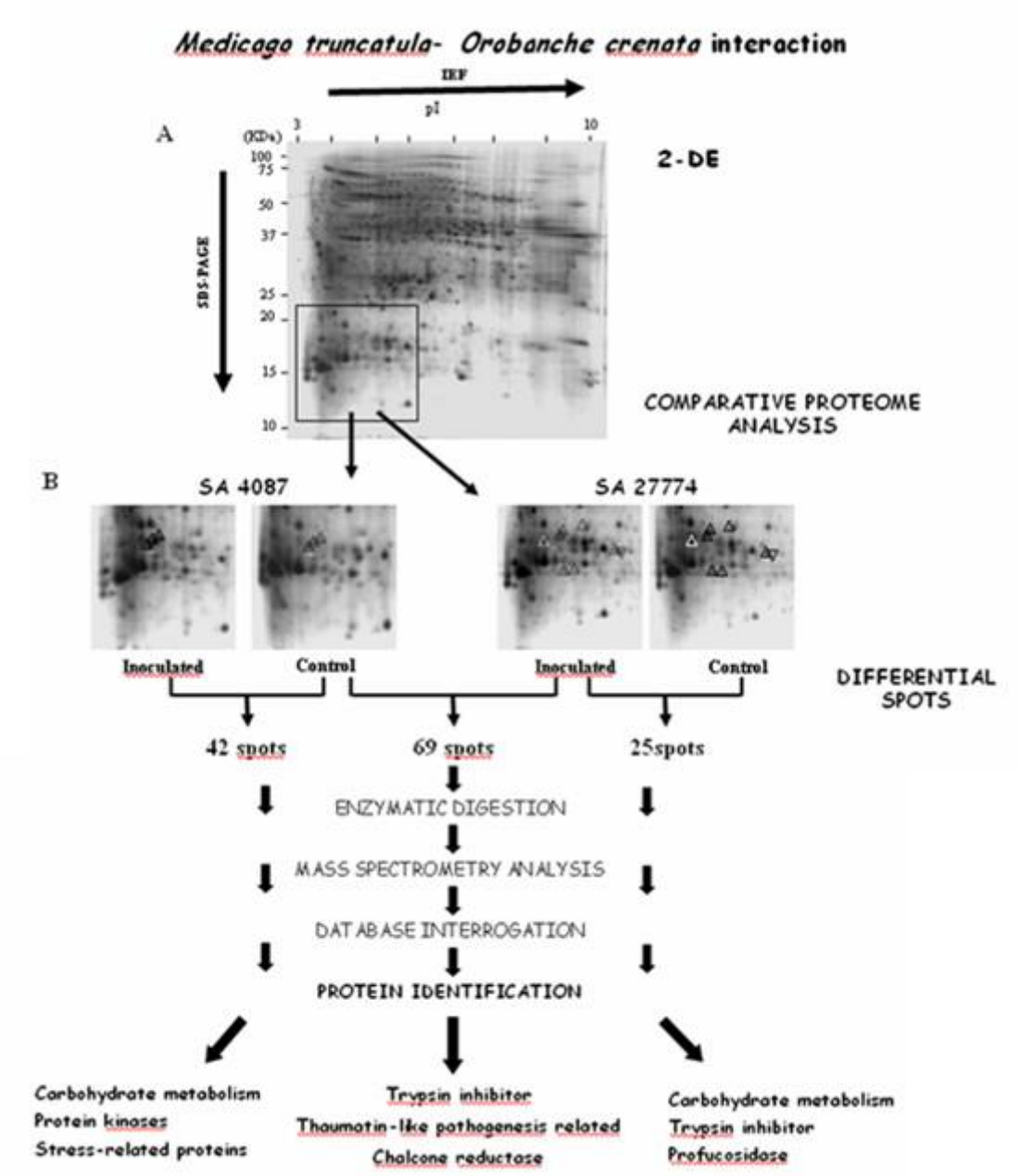

Figure 5: Schematic representation of the strategy utilized in the identification of proteins differentially expressed in leaves of *Medicago truncatula* in response to the inoculation with *Orobanche crenata.* A, 2-DE gel (pH 3-10) of infected leaf tissue. B detail of the gel area indicated in A showing differential expressed spots in non-inoculated and inoculated SA 4087 and SA 27777 plants. C. Excised spots are subjected to trypsin digestion, mass spectrometry analysis (MALDI-TOF). The peptide mass fingerprinting data were used to interrogate databases allowing protein identification.