Resistance to Rusts (*Uromyces pisi* and *U. viciae-fabae*) in Pea

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Abstract

Barilli E., Sillero J.C., Prats E., Rubiales D. (2014): **Resistance to rusts (*Uromyces pisi* and *U. viciae-fabae*) in pea**. Czech J. Genet. Plant Breed., **50**: 135–143.

Pea is the second most important food legume crop in the world. Rust is a pea disease widely distributed, particularly in regions with warm, humid weather. Pea rust can be incited by *Uromyces viciae-fabae* and by *U. pisi*. *U. viciae-fabae* prevails in tropical and subtropical regions such as India and China, while *U. pisi* prevails in temperate regions. Chemical control of rust is possible, but the use of host plant resistance is the most desired means of rust control. In this paper we revise and discuss the occurrence and incidence of both pathogens on peas, the availability of resistance sources and the present state of the art in pea breeding against this disease.

Keywords: breeding; histology; *Pisum sativum*; resistance; rust

Grain legumes are important crops which can decrease the marked deficit of high-protein feedstuff and contribute to a large extent to the sustainability of crop-livestock systems (Carrouée *et al*. 2003; Annicchiarico & Iannucci 2008). Among them, dry pea (*Pisum sativum* L.) is the second most important food legume crop in the world because of its high yield potential (Annicchiarico & Iannucci 2008; Rubiales *et al.* 2011a; Smýkal *et al.* 2012).

Pea rust has become an important pathogen of dry pea since the mid-1980s and is mostly distributed in Europe, North and South America, India, China, Australia and New Zealand, particularly in regions with warm, humid weather (EPPO 2012). The pathogen usually appears during mid-spring when the crop is at flowering or podding stage. In years of epidemics, affected leaves dry up and fall off, and pods remain undeveloped, which consequently results in yield losses of higher than 30% (EPPO 2012). Chemical control of rust is possible (Singh *et al.* 2004; Emeran *et al.* 2011), but the use of host plant resistance is the most desired means of rust control (Rubiales *et al.* 2011a).

Pea rust has been reported to be caused either by the fungus *Uromyces viciae-fabae* (Pers.) J. Schröt [syn. *U. fabae* (Pers.) de Bary] (Pal *et al*. 1980; Xue & Warkentin 2001; Singh *et al*. 2004; Vijayalakshmi *et al*. 2005; Kushwaha *et al*. 2006) or by *U. pisi* [(Pers.) Wint.] (Emeran *et al*. 2005; Barilli *et al*. 2009a, b). The species complex *U. viciae-fabae*, commonly referred to as faba bean rust, is an autoecious fungus reported to infect pea besides faba bean (*Vicia faba* L.), lentil (*Lens culinaris* L.) and common vetch (*Vicia sativa* L.) (Cummins 1978). Isolates of *U. viciae-fabae* are specialized with respect to their hosts, with each isolate exclusively infecting cultivars of the species from which it was collected (Emeran *et al*. 2005; Rubiales *et al.* 2013). *U. viciae-fabae* is the principal causal agent of pea rust in tropical and subtropical regions like India and China, where warm humid weather is suitable for the appearance of both the uredial and the aecidial stage (Kushwaha *et al*. 2006). However, in temperate regions, it has been observed that although pea seedlings can be infected by *U. viciae-fabae*, it barely gets established and progresses under field conditions slowly (Barilli *et al*. 2009c). These observations were confirmed by gathering several rust isolates from highly damaged pea crops from different geographical regions (Canada, Czech Republic, Egypt, Morocco and Spain). Molecular analyses confirmed that all isolates belonged to *U. pisi* rather than to *U. viciae-fabae* (Barilli *et al*. 2011). *U. pisi* is a heteroecious macrocyclic fungus that completes its life cycle on the spontaneous

Euphorbia cyparissias (cypress spurge) (Pilet 1952), over the vegetation residues of which the fungus overwinters as teliospores (Pfunder & Roy 2000). Spermatogonia and aecia develop on this alternate host, while the pathogen produces several generations of urediniospores on pea leaves and stems. In India it has been reported that *U. viciae-fabae* aeciospores act as repeating spores and play an important role in the outbreak of the disease (Kushwaha *et al*. 2006), whereas in temperate regions urediospores are the only infecting spores in the case of *U. pisi* (Xue & Warkentin 2001).

The *U. pisi* host range is wide, being able to affect plant species from many other genera (*Astragalus*, *Cicer*, *Euphorbia*, *Lathyrus*, *Lens*, *Medicago*, *Orobus*, *Pisum* and *Vicia*, among others) (Barilli *et al.* 2012a; Farr *et al*. 2008; Vaz Patto *et al*. 2009a). *U. viciae-fabae* is circumglobal on *Lathyrus*, *Pisum* and *Vicia*, with *V. faba* the host of the neotype (Cummins 1978). However, host-specialized isolates that cannot infect *V. faba* have been reported (Emeran *et al.* 2005, 2008; Rubiales *et al.* 2013). In addition, the pathogen has been described infecting plants from the genera *Ervum*, *Lens*, *Melilotus* and *Orobus* (Farr *et al*. 2008).

Screening and sources of resistance

Many efforts have been made to identify sources of resistance in pea against *U. viciae-fabae* (Pal *et al*. 1980; Xue & Warkentin 2001; Singh *et al*. 2004; Vijayalakshmi *et al*. 2005; Kushwaha *et al*. 2006). These studies revealed that the majority of the studied pea genotypes were susceptible, though genotypic differences in the rust intensity described as of slow rusting type have been reported (Kumar *et al.* 1994; Chand *et al.* 2006) (Table 1). Slow rusting resistance against *U. viciae-fabae* has been characterised by measuring the area under the disease progress curve (AUDPC), the disease severity (DS), the number of pustules/leaf or infection frequency (IF) and the pustule size (Chand *et al.* 2006). The authors considered that AUDPC values were more precise for genotype selection than DS.

An exception was reported for the accessions PJ22211, PJ207508, EC109188, which were found to be immune to *U. viciae-fabae* infection (Pal *et al*. 1979), while in F_2 and backcross generation there were symptoms of hypersensitive reaction to the pathogen that were typified by the presence of large numbers of minute brown islands on the leaves.

Little work has been performed with *U. pisi* resistance in *Pisum* and only recently a pea germplasm collection has been screened to identify sources of resistance to this pathogen both under field and growth chamber conditions (Barilli *et al*. 2009b) (Table 1). Different epidemiological parameters, such as DS and AUDPC (as mentioned above), as well as infection type (IT), epidemic growth rate (r) and time of the first pustule appearance (t_0) were investigated, as well as the relationship between them, in order to identify the parameters that better characterize the resistance to *U. pisi* in both conditions. No complete resistance has been identified so far. However, incomplete resistance was common in the collection, with more than 60% of the accessions showing markedly lower severity values than the susceptible check. All the accessions displayed a compatible interaction (high infection type) both in adult plants under field conditions and in seedlings under growth chamber conditions, but with varying levels of disease reduction (Barilli *et al*. 2009b) (Figure 1), suggesting the existence of partial resistance *sensu* Parlevliet (1983). The correlation observed between DS values measured under field conditions during three growing seasons, and between DS and AUDPC was high (Barilli *et al*. 2009b) suggesting that the final DS estimation on pea provides a feasible estimation of partial resistance. DS estimation

Table 1. Reported sources of pea resistance to *Uromyces viciae-fabae* and *U. pisi*

Disease	Source of resistance	Gene related	References
U. viciae-fabae	Bridzor,EC4294, EC9218, EC955 EC9908, NP29, Perf 3268, IC4604		Sohi *et al*. (1974)
	PJ207508, PJ22211, EC109188	monogenic inheritance	Pal *et al.* (1979, 1980)
	RPB-22, RA-10-5, RC-35-2		Abusaleha and Pal (1990)
	Pant P11, FC1, HUDP 16, JPBB 3, HUP 14	monogenic (putative *Ruf)* inheritance	Chand *et al.* (2006); Kushwaha *et al.* (2006)
U. pisi	IFPI3260, PI347321, PI347336, PI347347, PI343935, PI343965, PI347310	inheritance under study	Barilli *et al.* (2009a, b)

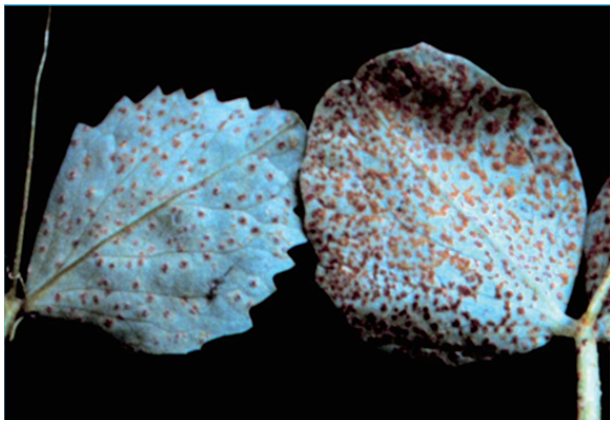

Figure 1. Symptoms observed two weeks after *Uromyces pisi* inoculation on pea susceptible (Messire, right) and partially resistant (PI347321, left) genotypes

needs less computation and is less time-consuming than assessing AUDPC, epidemic growth rate (r) or the first pustule appearance (t_0). The epidemic growth rate and the first pustule appearance were poor estimators of *U. pisi* partial resistance as they were less discriminating than the other parameters and showed a low correlation within experimental designs.

Growth chamber studies have shown that DS values are higher on the abaxial than on the adaxial leaf surface. The vertical shape of a *U. pisi* substomatal vesicle that penetrates deeper into the leaf mesophyll should be the cause of this finding (Emeran *et al.* 2005). There was a good correlation between DS measured in the growth chamber and adult plants in the field, proving that selection for partial resistance to pea rust can be effectively performed in seedlings.

Lack of durability of resistance is a problem of airborne fungal pathogens like rusts. Several mechanisms may prevent the rust infection prior to stomatal penetration (Rubiales & Niks 1996; Sillero & Rubiales 2002; Sillero *et al.* 2006; Prats *et al.* 2007a; Rubiales *et al.* 2011b). These can include limited germination or germling adhesion to the leaf surface (Mendgen 1978) and/or altered stomatal guard cell morphology (Wynn 1976). The limited varietal differences in spore germination observed in the *U. pisi*-pea pathosystem agreed with previous reports in which reduction of urediospore germination and fungal development on the leaf surface are of marginal importance in reducing infection levels within the host species (Niks & Rubiales 2002). In general, most resistance mechanisms to rust infection occur after the formation of substomatal vesicles, before or after mesophyll cell penetration. A relatively high proportion of germlings failing to form any haustoria in mesophyll cells causing "early aborted colonies" has been observed in some pea accessions (Table 2). Probably, epidermal cells developed papillae and/or cell wall strengthening under the site of the attempted attack avoiding the fungus penetration, as described previously in other plant-rust interactions (Prats *et al.* 2007b; Rojas-Molina *et al.* 2007). Further studies are currently carried out to study host cell

Table 2. Reaction of selected accessions of *Pisum* spp. to inoculation with *Uromyces pisi* under growth chamber conditions (according to Barilli *et al.* 2009a)

Accession	Species	Macroscopical components of resistance			Microscopical components of resistance		
					2 DAI		6 DAI
		IT	LP (%)	IF	early abortion (%)	No. of haust/ colony	colony size (mm^2)
Messire (check)	*P. sativum*	4	100	100.0	0.0	10.8	0.6
PI347321		4	108*	25.9*	10.5*	3.5*	0.38*
PI347336		4	106*	30.9*	3.5*	6.4*	0.38*
PI347347		4	104*	30.2*	6.0*	5.2*	0.41*
PI343965		4	104*	30.9*	0.0	6.3*	0.36*
PI347310		4	104*	29.5*	0.0	4.2*	0.41*
IFPI3260	*P. fulvum*	4	107*	13.7*	20.0*	2.2*	0.26*

*Significantly different from Messire (LSD test, P <0.01); IT – infection type according to Stackman *et al.* (1962) scale; LP – latent period measured as period of time (h) between inoculation and sporulation of 50% of the pustules; values are presented as % with respect to the susceptible Messire (= 200 h); IF – infection frequency measured as number of pustules per cm^2; values are presented as % with respect to the susceptible Messire (= 139 pustules/cm^2); DAI– days after inoculation

wall modifications in response to *U. pisi*. In several resistant genotypes the first haustorial mother cells succeeded in penetrating the mesophyll cell and forming a haustorium, subsequent penetration attempts by secondary hyphae failed, reducing the number of haustoria per colony and therefore hindering the number of hyphal tips and the growth of the colony (Barilli *et al.* 2009a). A similar resistance mechanism was found in *Lathyrus sativum* and *L. cicera*, *Medicago truncatula*, wheat, garlic, chickpea and faba bean against *U. pisi* (Vaz Patto *et al.* 2009a, b), *U. striatus* (Rubiales & Moral 2004; Rubiales *et al.* 2011b), *Puccinia triticina* (Rubiales & Niks 1995), *P. allii* (Fernández-Aparicio *et al.* 2011), *U. ciceris-arietini* (Sillero *et al.* 2012) and *U. viciae-fabae* (Sillero *et al*. 2000; Sillero & Rubiales 2002), respectively. All the selected accessions to *U. pisi* showed a critical decrease in the number of hyphal tips per colony compared to the susceptible check suggesting that haustorial mother cells have been formed but are not functional or that they have a reduced ability to successfully develop a haustorium in the plant cell, as found in faba bean against *U. viciae-fabae* (Rubiales & Sillero 2003). Thus, penetration resistance is an important mechanism to prevent the full development of *U. pisi* infection structures. This resistance is initially expressed with the arrest of the infection by early abortion, and continued by hampering subsequent haustoria formation. Macroscopically, penetration resistance resulted in smaller colonies that developed more slowly than the susceptible control as reflected by the negative correlation observed between latent period (LP) and colony size (CS) at any day after inoculation.

When the haustoria invade host cells, hypersensitive response (HR) can be triggered. The HR is often mediated by the genetic interaction of a host-encoded resistance (*R*) gene product with that of a pathogen avirulence (*avr*) gene leading to programmed cell death, thus limiting fungal development (Sillero & Rubiales 2002). The HR is common in biotrophic pathogen-plant interactions and was described in cereal and legume responses to rust, among others (Tiburzy & Reisener 1990; Sillero & Rubiales 2002; Shtaya *et al*. 2006). HR may be triggered early or late depending on the specific host genotype. However, we did not observe any HR in pea against *U. pisi*. Resistance was not associated with host cell death 2 days after inoculation (DAI) in any of the accessions studied (Barilli *et al*. 2009a), discarding the fast HR hypothesis. Neither was host cell death observed later (6 DAI) (Barilli *et al*. 2009b) discarding also the possibility of late-acting HR that was reported in other interactions such as *L. cicera-U. viciae-fabae* (Vaz Patto *et al.* 2009b), *V. faba-U. viciae-fabae* (Herath *et al*. 2001; Sillero & Rubiales 2002) and barley-*Puccinia hordei* (Niks 1986). Incomplete resistance identified in pea against *U. pisi* is not therefore based on HR, fitting the definition of Partial Resistance (PR) (Parlevliet & Van Ommeren 1975). Similarly, PR against rusts has also been reported in other legumes such as faba bean (Sillero *et al*. 2000; Herath *et al*. 2001), common bean (Statler & McVey 1987), grass pea (Vaz Patto *et al.* 2009a), chickling pea (Vaz Patto *et al.* 2009b) and chickpea (Madrid *et al*. 2008; Sillero *et al.* 2012).

The fact that the studied resistant accessions showed pre-penetration resistance offers breeding opportunities for this trait. This is important since penetration resistance is usually non-race dependent and based on multiple genes. Thus, such resistance is expected to be more durable than single gene controlled race-specific resistance that, although easily manipulated in plant breeding, is also easily overcome by new races of pathogens. Accessions IFPI3260 and PI347321 have been included in our breeding programmes and their molecular bases of resistance to *U. pisi* are under study.

Inheritance of resistance

To date, studies on the genetic basis of resistance to *U. viciae-fabae* have indicated either monogenic (Pal *et al*. 1979; Katiyar & Ram 1987) or polygenic control (Kumar *et al.* 1994; Vijayalakshmi *et al*. 2005). The dominant nature of partial resistance against faba bean rust *U. viciae-fabae* in pea, recorded as reduced infection frequency, has been justified as the expression of a single major gene, for which the symbol *Ruf* was proposed (Vijayalakshmi *et al*. 2005), but the authors also presented some evidence suggesting involvement of some polygenes as well. Further, none of the pea genotypes has been reported to be free from *U. viciae-fabae* infection (Singh & Sivastava 1985; Chand *et al.* 2006) suggesting a polygenic type of resistance or based on incomplete gene expression.

More recently, Rai *et al*. (2011) suggested that the *Ruf* gene proposed by Vijayalakshmi *et al*. (2005) be now redesigned as *Qruf* to signify the quantitative nature of its action and detected another minor quantitative trait loci (QTL) (named *Qruf1*). Both QTLs were located on LGVII. *Qruf* was flanked by SSR markers, AA505 and AA446 (10.8 cM), explaining

22.2–42.4% and 23.5–58.8% of the total phenotypic variation for IF and AUDPC, respectively. *Qruf* was consistently identified across four environments. Therefore, the SSR markers flanking *Qruf* would be useful for marker-assisted selection for *U. viciae-fabae* resistance. The minor QTL was environment-specific, and it was detected only in the polyhouse (logarithm (base 10) of odds values 4.2 and 4.8). It was flanked by SSR markers, AD146 and AA416 (7.3 cM), and explained 11.2–12.4% of the total phenotypic variation.

In pea, two segregating populations derived from the crosses between a resistant and a susceptible accession of *P. fulvum* L. (IFPI3260 × IFPI3251) and of *P. sativum* (PI347321 × Messire) have recently been developed to study the resistance against *U. pisi*. In an early study using the IFPI3260 × IFPI3251 cross, F_2 plants were evaluated in the field and F_3 families under growth chamber conditions assessing DS and IT values as mentioned above. A wide range of disease reactions was found in the population, although high IT values, indicating the absence of hypersensitive response, were observed on all the lines. Preliminary results on this population revealed polygenic inheritance. A single QTL was detected, *UP1*, located between markers OPY11_1316 and OPV17_1078 to govern the resistance of *P. fulvum* accession IFPI3260 to *U. pisi* under controlled conditions, although there was a hint of a second QTL between markers OPAB12_125 and OPY11_1361. This QTL, *UP1*, explained up to 60% of the phenotypic variance (Barilli *et al.* 2010a).

A RIL (Recombined Inbred Line) population derived from this cross is being developed at present in order to perform the required replications of field tests, characterizing their effects and validating the stability of QTLs across environments. Besides, a RIL population derived from the cross PI347321 × Messire (*P. sativum* intraspecific cross) has also been developed and F_6 plants have been evaluated against *U. pisi* under both natural and controlled conditions (unpublished data) and QTL analysis is under study in this moment.

Molecular markers linked to resistance genes could facilitate the selection of rust resistant segregants and thereby improve breeding efficiency. So far, reports on molecular mapping of resistance to *U. pisi* are limited and more robust markers are needed. But rust resistance breeding is not only slow due to these still insufficient genomic resources, but also, and mainly because of the little knowledge of the biology of various rust pathogens, it still lacks the knowledge of the basic aspects such as the existence of races and their distribution. Only after significant input to improve the existing knowledge of biology of the causal agents as well as of the plant, resistance breeding will be efficiently accelerated.

Induced resistance

In order to validate alternative pea rust control methods, a preliminary study on systemic acquired resistance (SAR) induction on this plant-pathogen interaction was developed using both biotic (*U. pisi* and *U. appendiculatus*) and abiotic (salicylic acid (SA), benzo-(1,2,3)-thiadiazole-7-carbothionic acid (BTH) and DL-β-aminobutyric acid (BABA)) inducers (Barilli *et al.* 2010b). Results obtained showed a significant reduction of infection levels locally and systemically with BTH and BABA foliar treatments, whereas neither biotic inducers nor SA had any significant effect hampering the rust development.

BTH is a chemical SAR inducer against a wide range of pathogens even though its effect varied with the concentrations used and the pathosystems considered (Van Loon 2001). The expression of BTH-induced SAR has been associated with transcriptional activation of gene encoding pathogenesis-related (PR) proteins promoted by endogenous accumulation of SA (Jiang *et al.* 2008). In the rust-sunflower and rust-wheat interaction, BTH-induced SAR has also been associated with the excretion of phytoalexins to the leaf surface, which inhibited urediospore germination and appressorium formation (Prats *et al.* 2002).

The cellular and molecular mechanisms through which BABA exerts its action are not so well reported as those of BTH. Also, its capacity to confer protection against basidiomycetes in general, and rusts in particular, is contradictory (Amzalek & Cohen 2007). In sunflower, unlikely BTH, BABA does not seem to induce any inhibitory effect on *Puccinia helianthi* on the events prior to stomatal penetration (Amzalek & Cohen 2007), suggesting that the resistance induced by these two chemicals operates via different pathways.

To clarify the underlying mechanisms acting in the BTH and BABA-induced resistance in pea against *U. pisi*, the specific enzymatic activity enhanced in a susceptible and in a partially resistant pea genotype was studied (Barilli *et al.* 2010c). The disease reduction observed after treatment with the inducers was not complete. Treatment with 10mM BTH and 50mM BABA effectively reduced the infection frequency, with this reduction being higher in the partially resistant than in the susceptible genotype.

The reduction in IF cannot be attributed to the toxic effect of the chemicals on the fungus, as neither of them showed a fungistatic activity against *U. pisi* urediospores. Furthermore, the observed protective effect was related to triggering of defence responses, as reported for other plant-pathogen interactions (Prats *et al*. 2002; Iriti & Faoro 2003; Amzalek & Cohen 2007).

Resistance was characterized by reduced infection frequency mainly due to decreases in appressorium formation, stomatal penetration, growth of infection hyphae and haustorium formation. Changes in β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase and peroxidase activities and in total phenolic content demonstrate that *U. pisi* resistance is induced by BTH and BABA treatments at early and late stages of the fungal infection process, but that the chemicals operate via different mechanisms. In fact, we should observe that BTH treatment primed the activity of pathogenesis-related proteins such as β-1,3-glucanase, chitinase and peroxidase in both susceptible and resistant genotypes. On the other hand, BABA treatment did not increase the enzymatic activities in the studied genotypes, but significantly increased their total phenolic contents. This increase was also observed in BTH treated plants. In addition, preliminary results showed differences in the amount and nature of particular phenolic compounds, excreted to the leaf surface following the treatment with both inducers (unpublished). This suggests a role for phenolic compounds in the induced resistance exerted by both BTH and BABA. It has been well documented in different pathosystems that phenolic compounds can play an important role in disease resistance, limiting fungal germ tube development or appressorium formation and contributing to the cell wall strengthening (lignins), thus preventing the plant tissue colonisation (Prats *et al*. 2002). The induction of the phenolic biosynthesis pathway by both inducers might therefore actively contribute to the resistance to *U. pisi*. In order to confirm this hypothesis, a proteomic approach was applied (Barilli *et al*. 2012b). Two-dimensional electrophoresis (2-DE) was used in order to compare the leaf proteome of the susceptible and the partially resistant pea genotypes in response to parasite infection under the effect of BTH and BABA. Multivariate statistical analysis identified 126 differential protein spots under the experimental conditions (genotypes/treatments). All of these 126 protein spots were subjected to MALDI-TOF/TOF mass spectrometry to deduce their possible functions. A total of 50 proteins were identified using a combination of peptide mass fingerprinting (PMF) and MSMS fragmentation. Most of the identified proteins corresponded to enzymes belonging to photosynthesis, metabolism, biosynthesis, binding and defence response, whose behaviour pattern was different in relation to susceptibility/resistance of the studied genotypes and to the BTH/BABA induction to pathogen response. Results obtained in this work suggested that plants could reduce their photosynthesis and other energy metabolism and enhance the production of defence-related proteins to cope with the stress. On the other side, we postulated that resistance induced by the chemicals operates via different mechanisms: BABA inducer could act via phenolic biosynthesis pathway, whereas resistance provided by BTH inducer seems to be mediated by defence and stress-related proteins. These results provide a step to understand the molecular basis of the induced resistance to rust in pea. Nevertheless, a higher collection of candidates will be essential to elucidate target key elements involved in SAR response using integrated studies.

CONCLUSIONS

Pea rust is a serious disease of pea of the worldwide distribution. Although no completely effective source of resistance has been found, considerable progress has been made in identifying germplasm with moderate levels of resistance. The effectiveness of these incomplete levels of resistance in reducing *U. pisi* infection remains to be quantified, but might represent a major progress when compared to the lack of any means for the control of this rust one or two decades ago. Peas can be protected now by combining this resistance with cultural management options, selective fungicides and by biocontrol agents representing opportunities that did not exist before.

The current focus in applied breeding is taking advantage of biotechnological tools to develop more and better markers to allow marker-assisted selection with the hope that this will accelerate the delivery of improved cultivars to the farmer. Our understanding of the genetics of resistance to pea rust in the available germplasm has improved considerably, but progress in marker development and delivery of useful markers is still limited. We are currently facing an accelerated progress in genomic and biotechnological research, which should soon provide important understandings on pathogen-host interactions and will provide candidate genes for resistance to pea rust. The effectiveness of MAS might soon

increase with the adoption of new improvements in marker technology together with the integration of comparative mapping and functional genomics. For this reason, the new genome-wide approach is emerging as a powerful tool for identifying quantitative characters, and its application to *U. pisi* resistance offers a significant potential.

Comprehensive studies on the host status and virulence of causal agents are often missing being a major limitation for any breeding programme. Only after a significant input to improve the existing knowledge of the biology of causal agents as well as of the host plant, resistance breeding will be accelerated efficiently.

Acknowledgements. The authors would like to thank projects AGL2011-22524, co-financed by FEDER for financial support and to Ana Moral for technical assistance along the years. Eleonora Barilli is postdoc funded by I3P program.

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Receieved for publication June 16, 2013
Accepted after corrections November 27, 2013

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