SITE OF INOCULATION AND STAGE OF PLANT DEVELOPMENT DETERMINE SYMPTOM TYPE AND EXPRESSION IN *BRASSICA JUNCEA* FOLLOWING INFECTION WITH *ALBUGO CANDIDA*

P. Kaur[1], K. Sivasithamparam[1] and M.J. Barbetti[1,2]

[1] School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, Crawley, WA 6009, Australia
[2] The UWA Institute of Agriculture, Faculty of Natural Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

SUMMARY

Investigations were carried out firstly to determine if there were any differences in symptom expression following spot inoculation of the cotyledons, leaf lamina or the growth apex at different stages of plant growth and, secondly, to determine the specific reproductive stage during development of *Brassica juncea* at which the flowers have to be inoculated with *Albugo candida* to maximise development of pod hypertrophies. Spot and growing point inoculations performed at the cotyledonary or true leaf stages caused clear differences in disease progression and severity of disease occurring at subsequent stages of plant growth. In this test, inoculations of only the growing points at the true leaf stage [GS 2.1] resulted in systemic pod hypertrophies. However, among the different reproductive stages tested, only inoculations of flower buds just changing colour [GS 3.3] or just opening flowers [GS 4.1] resulted in pod hypertrophies. Earlier or later inoculations of flower buds or flowers failed to result in pod hypertrophies. These studies have defined, for the first time, the particular reproductive stages of plant development at which infection results in pod hypertrophies. They have also shown that, in relation to infections of shoot tissues, it is the inoculation of the growing point at the first true leaf stage that leads to systemic development of the disease, rather than inoculations of cotyledons or leaf laminae. The findings of this study indicate that fungicide applications, targeting these critical stages of early plant development, and the associated plant sites, and also flower buds changing colour and/or just opening, present the best opportunities for arresting infections that lead to systemic spread of the pathogen within the plant, thus minimizing the extent and severity of both leaf disease and pod deformations. These studies provide a basis for subsequent identification of the mechanisms controlling the systemic spread of *A. candida* within *B. juncea*. Once identified, such mechanisms could be exploited in developing and deploying new cultivars with improved resistance against both white rust leaf disease and pod hypertrophies.

Key words: White blister rust, symptom expression, pod hypertrophy, staghead.

INTRODUCTION

White rust, caused by the oomycete pathogen *Albugo candida*, is a serious disease of various Brassicaceae species worldwide (Lakra and Saharan, 1989), including Western Australia (Barbetti, 1981; Barbetti and Carter, 1986; Li *et al*., 2007a, 2008). This disease is characterized by the formation of white to cream coloured zoosporangial pustules on cotyledons, leaves, stems and inflorescences, with deformation and/or stagheads formed as the result of inflorescence infection (Verma and Petrie, 1980). Yield losses of 30-60% have been reported in severely infested fields of *Brassica rapa* in Canada (Bernier, 1972) and of up to 20 and 60% on the same species in Australia (Barbetti, 1981) and India (Lakra and Saharan, 1989), respectively. In India, combined infection of *B. juncea* leaves and inflorescences causes yield losses up to 90%, with up to 63% of this yield loss attributable to the high level of staghead formation in susceptible cultivars (Lakra and Saharan, 1989; Kolte, 1985).

White rust has become a concern in Australia as canola-quality *B. juncea* is now being utilized to extend oilseed *Brassica* production into lower rainfall areas of southern Australia. This species is better adapted to hotter and drier environments than canola (*Brassica napus*) (Downey, 1971; Woods *et al*., 1991; Oram *et al*., 2005). Dune, the first canola-quality *B. juncea* cultivar, has been released (Anonymous, 2007) with an estimated 40,000 ha sown in 2009 (Anonymous, 2009). Unfortunately, most of the cultivated varieties of *B. juncea* in Australia are highly susceptible to white rust (Burton *et al*., 1999).

A. candida causes both localized and systemic infections on plants of various cruciferous species. In localized infections, the pathogen mainly infects plant parts that contain chlorophyll (Sokhi and Khangura, 1992). With systemic infections, extensive distortion, hypertrophy, hyperplasia and sterility of the stems, leaves, leaf petioles and inflorescences can occur (Verma and Petrie,

Corresponding author: M.J. Barbetti
Fax: +61.8.64887077
E-mail: martin.barbetti@uwa.edu.au

1980). Liu and Rimmer (1993) succeeded in obtaining deformed inflorescences resulting in stagheads in 40% of inoculated plants. However, Lakra and Saharan (1989) and Bains (1991) were unable to produce deformed inflorescences following similar inoculations of flower buds of susceptible *B. juncea* plants. To address such inconsistencies, Goyal *et al*. (1996) undertook a study and reported that maximum staghead formation in 26-day-old [growth stage (GS) 3.1; Harper and Berkenkamp (1974)] *B. juncea* plants occurs in response to inoculating differentiating flower buds. In contrast, they found that inoculation of 35- and 45-day-old plants (GSs 4.1 and 5.0, respectively) produced fewer hypertrophies mainly in isolated flowers, while inoculation of 7- and 13-day-old plants (GSs 1.0 and 2.1, respectively) produced no hypertrophied flowers. However, Goyal *et al*. (1996) did not make inoculations at GSs 3.2 nor at GS 3.3.

Our investigations firstly defined the relative importance of the full spectrum of growth stages, from cotyledon stage to young pods (viz. GS 1.0, 2.1, 3.1, 3.2, 3.3, 4.1, 4.2 and 4.3), in terms of inoculation and subsequent disease development of white rust; and, secondly, defined symptom expression following inoculations of the leaf lamina *vs* the growing point at the seedling (GS 1.0) and first true leaf (GS 2.1) growth stages.

MATERIALS AND METHODS

A known highly susceptible variety of *B. juncea*, RH 819 (Li *et al*., 2007b), was selected and its high susceptibility further confirmed with two additional screening tests conducted under the same controlled environmental conditions as used previously by Li *et al*. (2007b). For all experiments, seeds were sown into steam-treated potting mix (composted pine bark:cocoa peat:river sand, 2:1:1) in plastic trays with 8 cells (2.5×2.5 cm), in a controlled-environment room maintaining 13/18°C night/day temperatures, with a 16 h photoperiod at a light intensity of 520 µE m^{-2} s^{-1}.

Pathogen inoculum. Cotyledons of *B. juncea* RH 819, with white rust pustules from infection with a single isolate of *A. candida* race 2V recovered from *B. juncea* (Kaur *et al*., 2008), were collected 10 days post-inoculation (dpi) and stored at -80 °C for future use. Fully expanded cotyledons (10 days after sowing), were inoculated using a drop-inoculation technique (Kaur *et al*., 2008) where a single 10 µl drop of a zoosporangial suspension was spotted onto the adaxial surface of each of the two lobes of each cotyledon. Plants were then subjected to 4 days of enhanced humidity (>95% RH) by placing the pots in a high-humidity chamber [10 litre plastic storage boxes (32×22 cm) covered with clear plastic cling wrap]. When required, the cotyledons with pustules were removed from storage, thawed and the zoosporangia dispersed in deionised (DI) water and filtered through cheesecloth to remove plant debris. The concentration of the zoosporangial suspension was adjusted using a haemocytometer to 10^5 zoosporangia ml^{-1} before application to plants.

Controlled environment experiments. Two experiments were set up to determine the development stages of the host most conducive to the establishment of *A. candida* systemic spread. The first experiment (Table 1) was set up to investigate the differences in symptom expression resulting from leaf lamina and growing point inoculations at the early growth stages GS 1.0 and GS 2.1 (Harper and Berkenkamp, 1974) of *B. juncea*, using four different treatments and with eight replications for each GS. Treatments were as follows: spot inoculation of the cotyledon lamina (GS 1.0); spot inoculation of the growing point at the cotyledon stage; inoculation of leaf lamina of the first pair of true leaves (GS 2.1); inoculation of the growing point of the first pair of true leaves. Inoculated plants were incubated as previously described. This experiment was repeated once (experiment 1a and 1b).

The second experiment (Table 2) was set up to determine the most suitable reproductive stages for inoculation that maximizes the number of pod hypertrophies formed. Spot inoculation of the inflorescence was conducted at six different growth stages: bud development (rosette stage when the inflorescence had just become visible, GS 3.1), young (before changing colour, GS 3.2) and mature buds (lower buds just changing to yellow colour, GS 3.3), young (at opening, GS 4.1) and mature flowers (4-day-old, GS 4.2) and pod development (petal senescence, GS 4.3). Following inoculations, plants were incubated as previously described.

Disease assessments. Disease severity at the cotyledonary stage was assessed 12 dpi on a 0–9 scale where 0 = no disease and 9 = >75% of cotyledon area covered with pustules (Li *et al*., 2007a). Similarly, for the inoculations done at true leaf stage, disease severity was assessed on the first two true leaves at 12 dpi, according to the percentage of leaf area covered with white rust pustules (Li *et al*., 2007b). Disease incidence following cotyledon or leaf inoculation was recorded as the number of plants out of the total number of those being assessed that showed the particular symptoms. The growth stage (GS) of the plants used was as defined by Harper and Berkenkamp (1974).

Disease severity for the second experiment was assessed 14 dpi by estimating the percentage of leaf area covered by white rust pustules on the leaves proximal to the flower buds on the branch on which the flowers were inoculated (Li *et al*., 2007b), as well as the mean number of pod hypertrophies for each plant by counting the total number of hypertrophied pods and the number of plants. Disease incidence was assessed 14 dpi by recording the total number of plants showing symptoms.

Table 1. Number of diseased plants (maximum possible = 8) and mean severity rating (0-9 scale) of localized symptoms, number of pod hypertrophies (maximum = 8) and mean number of pod hypertrophies/infested plant on *Brassica juncea* plants following inoculations of different tissues (cotyledon, or leaf lamina, or the growing point) and incubation under controlled environment conditions. The experiment was repeated once (1a and 1b).

Treatment	Experiment 1a				Experiment 1b			
	Localized symptoms		Pod hypertrophies		Localized symptoms		Pod hypertrophies	
	Disease incidence	Disease severity	No. of plants showing pod hypertrophies	Mean no. of pod hypertrophies/ infested plant	Disease incidence	Disease severity	No. of plants showing pod hypertrophies	Mean no. of pod hypertrophies/ infested plant
Spot inoculation on cotyledon lamina only (GS 1.0)	6	3.0 (1.49)	0 (0.61)	0 (0.61)	5	3.0 (1.30)	0 (0.61)	0 (0.61)
Spot inoculation on the growing point at the cotyledon stage (GS 1.0)	8	5.8 (2.40)	0 (0.61)	0 (0.61)	7	5.7 (2.26)	0 (0.61)	0 (0.61)
Spot inoculation on first true leaf lamina only (GS 2.1)	5	2.6 (1.27)	0 (0.61)	0 (0.61)	5	3.0 (1.35)	0 (0.61)	0 (0.61)
Spot inoculation on the growing point at first true leaf stage (GS 2.1)	6	7.0 (2.13)	4.0 (0.89)	4.0 (1.07)	6	7.3 (2.22)	5.0 (0.96)	1.8 (1.14)
P value		*P < 0.05*	*P < 0.001*	*P < 0.001*		*P < 0.05*	*P < 0.001*	*P < 0.001*
LSD[b]		(0.82)	(0.15)	(0.27)		(0.81)	(0.14)	(0.25)

[a] Data for statistical comparison are given in parentheses and have been $\sqrt{(x+0.375)}$ transformed.
[b] LSD = least significant difference at $P = 0.05$.

Experimental layout and data analysis. All experiments included water-only controls, and were arranged using a randomized complete block design. A single factor analysis of variance was conducted using GENSTAT (8th Ed, Lawes Agricultural Trust, Hertfordshire, UK). Fisher's least significant difference (L.S.D) at 95% significance level was used to test the differences among the different treatments. A $\sqrt{(x+0.375)}$ transformation was applied to raw data from both experiments as the data were not normally distributed. Significant differences among means were assessed by LSD tests. The correlation for disease severity and incidence at the different growth stages between experiments 1a and 1b was predicted by regression analysis.

RESULTS

In the first experiment, the treatments relating to the site of inoculation resulted in significant differences in symptom type and expression. Spot inoculations on the cotyledon lamina resulted in the appearance of disease symptoms only on the cotyledons, without disease progressing to the leaves (Table 1). However, inoculations of the growing point at the cotyledonary stage led to progression of the disease to subsequently emerging leaves. Inoculation of growing points at the first true leaf stage not only led to the most severe disease of the treatments tested in this experiment, but was the only treatment leading to pod hypertrophies.

These results suggest that it is the deposition of inoculum on the growing point of the plants at the true leaf stage in the field that is most likely to lead not only to the most severe disease symptoms but also to the pathogen spreading within the plant systemically leading to pod hypertrophies. Although spot inoculation on the first true leaf lamina led to the disease progressing onto subsequently emerging leaves, it did not result in the deformation of pods produced subsequently. There was significant and positive correlation for disease incidence (r=0.92, P<0.001, n=32) and disease severity (r=0.79, P<0.001, n=32) between experiments 1a and 1b.

Among the different reproductive stages tested in the second experiment, during the growth of the plants, only the inoculations of the young flowers at opening resulted in hypertrophies of the pods (Table 2). Our studies showed that inoculations at GS 3.3 and GS 4.1 were the most conducive for the development of pod hyper-

Table 2. Number of diseased plants (maximum possible = 6) and mean severity rating (0-9 scale) of localized symptoms, number of pod hypertrophies (maximum = 6) and mean number of pod hypertrophies/infested plant on *Brassica juncea* plants following inoculations of different tissues and incubation under controlled environment conditions.

Plant growth stage at inoculation	Localized symptoms		Pod hypertrophies	
	Disease incidence	Disease severity	No. of plants showing pod hypertrophies	Mean No. of pod hypertrophies/ infested plant
Bud stage (When inflorescence become visible at the centre of rosette) (GS 3.1)	6	5.0 (2.25)	0 (0.61)	0 (0.61)
Flowers: Prior to presence of yellow colour of the flower buds (GS 3.2)	4	8.0 (2.13)	0 (0.61)	0 (0.61)
Flowers: When flower buds are just changing to yellow colour (GS 3.3)	4	6.7 (1.74)	2.0 (0.80)	2.0 (0.91)
Flowers: Just opening flowers (GS 4.1)	3	4.3 (1.39)	3.0 (0.89)	2.3 (1.11)
Flowers: 4 days post opening (GS 4.2)	3	2.0 (1.02)	0 (0.61)	0 (0.61)
Young pods: Senescence of flower petals (GS 4.3)	2	3.0 (0.91)	0 (0.61)	0 (0.61)
P value		*P < 0.05*	*P < 0.05*	*P < 0.05*
LSD[b]		(0.97)	(0.20)	(0.38)

[a] Data for statistical comparison are given in parentheses and have been $\sqrt{(x+0.375)}$ transformed.
[b] LSD = least significant difference at P = 0.05.

trophies and that as the inflorescences aged beyond the just opening of flowers, the occurrence of pod hypertrophies ceased. There were significant differences in the severity of symptom expression among these six reproductive stages. Although the maximum disease incidence was recorded at GS 3.1 no pod hypertrophies were recorded on plants inoculated at this growth stage. While pod hypertrophies were recorded at GS 3.3 when flower buds are just changing to yellow colour, the mean number of pod hypertrophies/infested plant was not significantly different to treatments where no pod hypertrophies were recorded.

DISCUSSION

The present study showed that between the different vegetative growth stages (GS 1.0 and GS 2.1) tested, it was the deposition of inoculum on the growing point of the plants inoculated at the true leaf stage (GS 2.1) that in the field should lead not only to the most severe disease symptoms but also to the pathogen spreading within the plant systemically and leading to subsequent pod hypertrophies. In contrast, an earlier study by Goyal *et al*. (1996) found that inoculation of the apical meristematic region at GS 1.0 and GS 2.1 led to formation of hypertrophied branches but not to development of hypertrophied pods. However, Goyal *et al*. (1996) did not test if there were any differences in symptom expression and/or disease progression resulting from inoculation of the cotyledon/leaf laminae compared with inoculation of the apical meristematic region.

Goyal *et al*. (1996) found that inoculation of green (differentiating) buds (GS 3.1) led to more severe deformation of pods than when inoculations were carried out at subsequent stages of flowering, as we confirmed in the present study. Furthermore, Goyal *et al*. (1996) did not undertake inoculations at GS 3.2 nor at 3.3. The present studies showed for the first time that GS 3.3 and GS 4.1 are in fact the most conducive growth stages for the development of pod hypertrophies and also that inoculation beyond the just opening of flowers phase did not result in pod hypertrophies. Goyal *et al*. (1996) and Bains (1991) also obtained a significantly lower frequency of inflorescence hypertrophy in plants inoculated at GSs 4.1 and 5.0 compared to the growth stages inoculated before GS 4.1. Goyal *et al*. (1996) suggested that a possible reason for this could be that most meristematic activity had ceased beyond the site of infection and that the metabolic activities leading to the cellular hypertrophy had been switched-off prior to infection. They also observed that inoculations at the very early stages (GSs 1.0 and 2.1), failed to cause any pod hypertrophies and suggested that this could be due to the inability of the pathogen to reach the meristematic tissues or the actively dividing parenchyma cells in time to be able to cause pod hypertrophies. The lack of a relationship between incidence or severity of disease on *B. juncea* leaves and the incidence of deformation of inflorescences observed by Li *et al*. (2007a, 2007b) and Goyal *et al*. (1996), is likely a consequence of inoculation/infection of the growing point not occurring at either the first true leaf stage (GS 2.1), nor when flower buds are just-changing to yellow (GS 3.3) nor when flowers are just opening (GS 4.1).

Our study showed that inoculations of the growing point at the first true leaf stage lead to systemic development of disease and that infection of flower buds either just as they change to yellow colour or when they are at the early opening phase leads to the development of pod hypertrophies. Infection at these specific growth stages is most likely to lead to the most severe disease and consequently the greatest yield losses. This has significant implications in relation to understanding the epidemiology of this disease and for its effective management. For fungicide applications (e.g. Barbetti, 1978, 1988a, 1988b) to be effective, they now need to be targeted to these critical stages of early plant development, and the associated plant sites, and also to flower buds changing colour and/or just opening, as these present the best opportunities for minimizing infections that lead to systemic infections. This new understanding of the disease systemic spread within the plant could now also be exploited to identify the resistance factors/mechanisms in *B. juncea* associated with reduced systemic spread of the pathogen. These mechanisms could then be subsequently further exploited in developing and deploying new cultivars with improved resistance against both leaf disease and pod hypertrophies.

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