THE TOXIC EFFECTS OF OXALIC ACID PRODUCED BY SCLEROTINIA SCLEROTIORUM ON ORGANS, CELLS AND PROTOPLASTS OF RAPE (BRASSICA NAPUS)

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INTRODUCTION

Sclerotinia sclerotiorum (Lib) de Bary can attack a very wide range of plants (Purdy, 1979), and on oilseed rape (Brassica napus) in particular, serious economic losses can result from severe infection in China (Wu and Liu, 1989). A large number of cultivars, breeding lines, plant introductions and relative or wild Brassica species were evaluated using different inoculation techniques and conditions that simulated natural infection, all the accessions were susceptible, although a few plants of some lines appeared to be tolerant (Wu and Liu, 1990).

Cell and tissue culture offers a variety of techniques of value for the identification and selection of resistance (Brettel and Ingram, 1979). Oxalic acid, a phytotoxin produced by S. sclerotiorum has been purified and characterized (Overall, 1952; Maxwell and Lumsden, 1970; Rai and Dhawan, 1976). The screening techniques for resistance or tolerance using the toxin were developed in seedlings and cell suspensions of sunflower (Huang and Dorrell, 1978; Noyes and Hancock, 1981), and in bean leaves (Tu, 1985).

The studies reported here were initiated to determine if the toxin showed selective toxicity to organs, cells and protoplasts of oilseed rape with different susceptibility and if so, an evaluation was then made to determine the possibility of using this in vitro techniques to select for mutants resistant to the toxin at the level of the cell.

MATERIALS AND METHODS

Plant Materials

Brassica napus L. cvs. Hua No. 8 and Hua No. 13 (high susceptible), 821 and 083 (low susceptible) were used in this study and kindly provided by Institute of Crop Genetics and Breeding, Huazhong Agricultural University; and Institute of Oil Crops, Chinese Academy of Agricultural Science, Wuhan, China.

Preparations of Calli, Cells and Protoplasts

The seeds were germinated on 0.7% agar supplemented with 3% sucrose at 25°C for five days. The callus was initiated from hypocotyl explants according to Song et al (1987) on J1 medium containing 0.2 mg/L 2,4-D, 1 mg/L NAA and 2 mg/L 6-BA. Cell suspension cultures were obtained from callus on J1 liquid medium containing 0.5-1.5 mg/L NAA and 0.5 mg/L 2,4-D on a giratory shaker (120 rpm). The cells filtered through sieves were cultured in the same liquid medium for

investigation. The isolation of protoplasts was according to Chuong et al (1985).

Toxin Treatment

Concentrations of oxalic acid solution was distributed into twelve 10 ml vials, four of which were allotted to each cultivar. Primary leaves were exised and the petioles were immediately submerged in a Petri plate of water. Each leaf was assigned to a vial. The petiole was inserted into the vial with a layer of Parafilm to prevent evaporation and leaf them at 25°C for incubation. The percentage of the leaf area with brown-rot-like symptom was assessed dairly for 4 days by visual estimation (Tu, 1985).

Callus was divided into small uniform pieces (approx. 25±5mg fresh wt), weighted and three or four pieces were placed in Petri dishes containing the appropriate J1 medium with different amount of toxin. Final fresh weight of each inoculum was determined after an incubation period and subculture at 25°C. Fold increase in fresh weight was calculated by dividing the increase in weight by the initial weight.

Test droplets were placed in Petri plates (2 cm diam.) Each drop consisting of a mixture of 200 ul of whole cell suspensions or protoplasts, 200 µl oxalic acid and 10 µl of Evan's blue (0.05%). These were left at 25°C in normal roomlighting for 1 hour, then observed with a dissecting microscope. Plates were scored at different toxin concentrations for the living cells and protoplasts (Noyes and Hancock, 1981).

RESULTS

Disease development

Before starting experiment on selection against the oxalic acid toxin, it was investigated that the development of Sclerotinia sclerotiorum in oilseed rape (Brassica napus) cultivars (lines) was much slower in low-susceptible cvs 821 and 083 than in high-susceptible cvs Hua No. 8 and Hua No. 13. The histopathology and ultrastructure of the infection process showed that there were significant differences in the patterns of infection and spread of the pathogen among different levels of susceptible cultivar (lines), which included the types of appressorium, speeds of lesion development and active responses of the host (Wu and Liu, 1990).

The changes of the oxalic acid content, pH values in vitro or in infected tissues were parallelled to the growth of the pathogen or the development of the disease, and the bioassay showed the typical symptoms on rape tissues were induced by oxalic acid (Wu and Liu, 1989).

Effects of Oxalic Acid on Rape Leaves

Oxalic acid was tested over the range of concentrations from 2.5 to 100.0 mM. All the cultivars (lines) developed brown rot symptom in response to application of the toxin to the leaves through uptake of petioles, with the areas and

rates of symptom development varying according to the concentration applied and duration of feeding. Treatment with the lowest concentration did not produce typical rot symptom after 3 days, but concentrations high than 5.0 mM gave visible symptoms within 24 h. There were significant differences in sensitivity to toxin between high-and low-susceptible cultivars (lines) at given toxin concentration and treatment time (Table 1).

The sensitivity to oxalic acid of leaves declined with the development of plant, whereas, the significant differences betweer high- and low- susceptible cultivars (lines) existed during seedling, bud, flower and mature stages of plants.

Effects of Oxalic Acid on Callus Growth

The drastic inhibitory effect of oxalic acid on callus of rape was apparent a few days after culture on toxin-containing medium, and this toxic effect was not strictly specific because most calli become necrotic after a few days culture at high dosage of toxin. At a given concentration of the toxin inhibited the callus growth of high-susceptible cultivars (lines), cultures from low-susceptible ones survived and grew further on the selection medium (Table 2).

It was also found that few calli showed highly tolerant to oxalic acid with more than 2.5 mM concentration both in high- and low- susceptible cultivars (lines) after three to four passages on the toxin medium. With the increase of toxin concentration, callus which still survived and grew further was selected as tolerant or resistant. Regenerated plants from selected and control cultures were tested for disease resistance by artificial inoculation, most of selected regenerants showed an much increased resistance than within plants from control cultures.

Effects of Oxalic Acid on Isolated Cells and Protoplasts

Concentrations of oxalic acid was added to freshly isolated cells and protoplasts. Staining with Evan's blue revealed oxalic acid killed cells and protoplasts of all the four cultivars (lines). Freshly isolated cell could resistant or tolerant higher concentrations of toxin than protoplast, and the cells and protoplasts from high-susceptible cultivars (lines) were more sensitive and were killed by shorter exposures than those of low-susceptible cultivars (lines) (Table 3, 4). The differences between high- and low-susceptible cultivars (lines) were most pronounced during 2 days in culture and with the lower concentrations, but after 5 to 7 days, staining with Evan's blue revealed that nearly all the cells and protoplasts were dead.

It was also found in this study that few cells and protoplasts were tolerant or resistant to oxalic acid even in some combinations with the higher concentrations both from high- or low- susceptible cultivars (lines), nevertheless, no cell divisions of these tolerant or resistant cells and protoplasts were observed during the whole period in toxin-containing medium.

DISCUSSION AND CONCLUSION

In view of many reports that the role of oxalic acid in the pathogenesis of S. sclerotiorum may be manifold. Oxalic acid may have direct toxicity to host cells (Overell, 1952; Rai and Dhawan, 1976), it can also work synergistically with pectotylic enzymes (Maxwell and Lumsden, 1970) and affect the pH of the infected tissues (Lumsden, 1972). Our results with these in vitro screening tests were consistent with the field epiphytotic data on cultivar (lines) susceptibility to Sclerotinia in oilseed rape. The less disease-susceptible cultivars maintained higher tolerance levels to oxalic acid than the more susceptible ones, when treatments were applied to leaves, calli, cells and protoplasts.

A variation of sensitivity to the toxin could be observed in the calli, cells and protoplasts both from high-and low-susceptible cultivars (lines). In this case it was often correlated with alteration in genetic and/or physiological characters. This variability are probably the consequence of the spontaneous mutations and somaclonal variations originated during the in vitro growth, a necessity in order to establish whether the observed differences in sensitivity are truly genetic.

In conclusion, although oxalic acid is toxic to a broad spectrum of cultivars (lines), the difference found between high and low- susceptibility indicates that this toxin may be a suitable selective agent in searches for resistance to the fungus. Further work is currently underway to induce and select for novel resistant mutant using these in vitro techniques at the level of the cell.

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Table 1. Toxic Effects of oxalic acid on rape leaves

Cultivar (line)	Duration of feeding (h)	Concentration of oxalic acid (mM) 5.0	10.0	20.0	40.0	80.0
821	24	0.0(1)	3.2a(2)	10.6a	27.2a	51.4a
083		0.0	4.8a	11.3a	29.8a	59.6a
Hua No.8		3.4a	12.2b	33.4b	52.4b	86.4b
Hua No.13		3.7a	13.4b	36.8b	56.3b	82.3b
821	48	0.2a	6.3a	36.5a	53.2a	80.9a
083		0.3a	7.1a	39.4a	51.7a	81.4a
Hua No.8		6.8b	21.5b	69.3b	93.4b	98.4b
Hua No.13		7.1b	23.2b	72.1b	97.6b	100.0b

(1) Figures are the percentage of leaf affected, each figure is an average of three replications.
(2) Means within columns followed by a common letter are not significantly different at P0.05 according to Dancan's multiple range test.

Table 2. Toxic Effects of oxalic acid on the growth of rape callus

Cultivar (line)	Concentration of oxalic acid (mM) 0.0	0.5	1.0	5.0	10.0
821	4.5a(1)	3.6a	3.1a	0.8a	0.0
083	4.4a	3.9a	2.9a	0.4a	0.0
Hua No.8	4.3a	2.9b	1.4b	0.0	0.0
Hua No.13	4.6a	2.7b	1.1b	0.0	0.0

(1) Means of fold increase in fresh wt were obtained from weights of 10 inocula. Means in a column followed by the same letter are not significantly different at the 5% level according to Dancan's multiple range test.

Table 3. Toxic effects of oxalic acid on rape isolated cells

Cultivar	Concentration of oxalic acid (mM)						
(line)	0.2	0.4	0.6	0.8	1.0	2.0	4.0
821	100.0a(1)	98.1a	96.4a	83.2a	65.4a	49.2a	28.7a
083	100.0a	96.3a	96.1a	87.2a	67.3a	43.3a	26.5a
Hua No.8	98.2a	89.5b	77.9b	64.2b	34.2b	7.4b	0.0
Hua No.13	100.0a	84.3b	74.1b	60.7b	31.5b	3.1b	0.0

(1) Each value is the mean percentage of living cells (% contoal) from three replications after 24 hr. Values in a column followed by the same letter are not significant (Dancan's multiple range test, P=0.05).

Table 4. Toxic effects of oxalic acid on rape protoplasts

Cultivar	Concentration of oxalic acid (mM)				
(line)	0.2	0.4	0.6	0.8	1.0
821	98.2a(1)	90.2a	76.4a	50.3a	17.3a
083	98.0a	89.3a	72.6a	54.9a	19.6a
Hua No.8	87.2b	57.4b	21.7b	2.3b	0.0
Hua No.13	83.3b	49.2b	17.9b	2.1b	0.0

(1) Figures are mean survival percentage of each 500 protoplasts with three replications. Figures in a column followed by the same letter are not significantly different at 5% level according to Dancan's multiple range test.