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Study of plant microbe interaction in rapeseed mustard (*Brassica campestris*) and determination of Salicylic acid content (in normal and infected) plant

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Abstract
Resistance can be described as the inherent ability of a plant to prevent or restricts the establishment and subsequent activities of a potential pathogen. The resistances thus can systemic, long-lasting and effective against a broad spectrum of pathogen. This response is termed as 'systemic acquired resistance' (SAR). Development of resistance is correlated with the accumulation of salicylic acid (SA) as well as the expression of a number of pathogenesis –related (PR) proteins.

Keywords: Phytoalexins, Systemic Acquired Resistance, Peroxidase, moribund cells

Introduction
Phytoalexins are antimicrobial low molecular weight compounds (secondary metabolites) that are synthesized denovo following pathogenic attack. (Bailey and Mansfield 1982). Phytoalexins are chemically diverse, but a large number of them are products of shikimic acid (phenylpropanoid) pathway from which many plant secondary metabolites (flavonoides), lignin and anthocynanins are derived. Key early enzymes in this pathway include phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase, 4-Coumarate-coA lygase (4CL), and Chalcone synthase. Phytoalexiens are typically synthesized in moribund cells surrounding the site of infection and accumulate to high levels in dead and dying cells.

Salicylic acid (SA) a benzoic acid derivative has been suggested to be an endogenous signal for SAR as its exogenous application has been shown to induce resistance to a variety of bacterial fungal and viral pathogen. SA is shown to be an endogenous phloem mobile compounds that increases in concentration at the onset of SAR in cucumber and *Arabidopsis.* (Bowling *et al*. 1994)

Triggering of systemic response is accelerated by the host cell death caused by either HR or hypersensitive response or by disease development. Early physiological events of HR to pathogen/inducer and their correlation with signal generation for SAR include the generation of reactive oxygen species (ROS) such as hydrogen peroxide. Hydrogen peroxide and other ROS derived compound act as inducer of defense genes. The ROS may direct by kill the pathogen, induce cross linking of cell wall proteins and / or enhance lignin synthesis catalyzed by peroxidase, thus creating a physical barrier against invading pathogen.

Rapeseed mustard (*Brassica ca*mpestris) oilseed crops are important sources of edible oil in Indian diet. It belongs to family Brassicacae and is cultivated on 6.86 mha in Rabi season (Sep/Oct-Mar/Apr), in India respectively.

The major rapeseed mustard growing states are Haryana, Madhya Pradesh, Rajasthan and U.P., together contributing 80.0 and 80.7 percent to the total national hectare and production respectively. Rajasthan has the largest hectare (2.99mha) and production (2.47 mt) which corresponds to 45.4 and 42.9 percent of total rapeseed mustard cropped area and production.

The current production of rapeseed mustard in India is 6.94 mt. According to a most conservative estimate, the domestic demand and supply gap in the edible oil stands at 1.5 mt per annum, and it is estimated that 58 mt. Of this total oil-seed production, the share of rapeseed mustard would be around 24.2 mt. to produce an additional quantity of more than 17 mt. in about years, realizing that there is very little scope of horizontal expansion of the crop, only practical option is the vertical growth of the crop, i.e., increasing yield.

To achieve the target envisaged, one strategy which is proposed for increasing the production of rapeseed mustard is-

Adoption of technology package replacement of old/obsolete varieties, and management of insect-pests and diseases.

Pusa Jaikisan (BIO-902) of Indian mustard is the first somaclonal variety developed through tissue culture, which is one of the variety used for experimental work.

Alternaria brassicicola (Schw.) Wiltshire is the causal organism of the disease alternaria leaf spot of crucifers. The fungus attack mustard, cabbage, cauliflower and knoll-knoll. Spores and mycelium can also survive in diseased plant debris, conidia are disseminated by wind. Small dark colored circular lesions develop on leaves infected by *A. brassicicola*. Concentric rings may develop in lesions. Linear spots form on petioles, pods and stem.

Though the mechanisms of defense have been worked out in many plants but many economically important plants have been ignored. The proposed work aims to study the plant microbe interaction in Rapeseed mustard *(Brassica campestris* family Brassicacae), and the objectives will be determination of SA content in normal and infected plants using calorimetric analysis of the two varieties.

Materials and methods

Plant material and pathogen culture

a) Maintenance of plant system

Seeds of BIO-902 and PCR-15 variety of Brown sarson i.e., Rapeseed *(Brassica campestris*) var. brown sarson were procured from krishi vigyan Kendra. Seeds were surface sterilized with 0.1%HgCl2 and washed several (2-3 times) in sterile distilled water. Seeds were sown on sieved and oven sterilized garden soil in plastic pots (60). So that at least 10-13 plants could grow in each pot.

Rapeseed (*B. campestris* var. Brown Sarson) was maintained in green house conditions of temperature 26 degree Celsius and 60% humidity with 12 hours photoperiod.

For experimentation 20 days, and 25 days old plants were chosen for infection in vivo, and same age plant parts (leaves) were harvested for in vitro estimation and quantitation.

Maintenance of fungal (pathogen culture)

The conical flask (250 ml capacity) containing sterilized PDA (Himedia) were UV sterilized. Under proper aseptic conditions, in laminar flow hood (YORKO Horizontal, New Delhi). Lyophilized fungal strains were rejuvenated under proper sterilization conditions and cultured in PDA medium at 28 degree C with aerobic conditions (G 24 Environmental incubator shaker, New Brunswick Scientific Co.Inc. Edison, N.J., USA), initially with 120 rpm speed in incubator shaker. Periodic sub culturing in PDA slants was done at intervals of 15-20 days.

Lyophilized *Alternaria brassicicola* (Schw.) Wiltshire (MTCC No. 2102) were obtained from IMTECH, Chandigarh. Strain was activated on PDA medium (potato infusion 200g/lt., dextrose 20g/lt., agar 15g/lt; PH-5.6+/-0.2 at 25 degree C.) autoclaved at 121 degree for 15 lb pressure for 15 MIN.

Preparation of Spore Suspension and Plant Infection

Spore suspension was prepared by the following procedure.

The surface of culture mat was scrapped gently with the sterilized inoculation loop under proper aseptic condition i.e., under LAF. Spore suspension was then adjusted to a concentration of 10,0000 spores/ml using haemocytometer. Plants of 20 and 25 days old were selected for inoculation. For in vitro experimentation, plant part (leaves) was kept in Petri-plates, layered with tissue paper. Surface epithelium was injured mildly with abrasives to facilitate pathogen entry. In both, in vivo and in vitro, systems were prepared in duplicates, i.e., normal (control or healthy) and infected (inoculated with pathogen). For infection, plants were sprayed with fungal spore suspension (prepared in sterile distilled water) using TLC sprayer.

Determination of Salicylic Acid

Colorimetric analysis

Method for the determination depends on the purple colour produced by the reaction of ferric ions with the phenol group in SA. In the test tubes, 1.0 ml of 75% methanol (10 ml/g tissue) extract of 20 days and 25 days old Rapeseed (*B.campestris* var. brown sarson), leaves after 0,4,24,48,72 hrs of inoculation was taken. 1 ml of distilled water served as blank and 1 ml SA was used as standard. To all tubes, 5 ml colour reagent was added and mixed vigoursly. Then the samples were spun at 2000rpm for 2 min before transferring each supernatant fluid to a cuvette using pasture pipette. Absorbance of standard was used to calculate amount of SA present in samples in mg/lt.

Standard solution of (200mg/l) of SA was prepared. Colour reagent was prepared dissolving 40g of HgCl2 in 850 ml H2O followed by addition of 120 ml HCl and 40 g Ferric nitrate (1 ml/lt.) and then volume was adjusted 1 lt. with water.

Results and discussion

The seeds of two varieties Bio-902 and PCR-15 procured from Krishi Vigyan Kendra, were surface sterilized with 01%HgCl2 and were sown in the plastic pots in oven sterilized soil. Among the two varieties, Bio-902 germinates faster than PCR-15. Two different age groups of plants (20 d and 25 d) were taken for studying the plant-microbe interaction.

Experiments were done by taking both intact leaves (in vivo) and excised leaves (in vitro) for comparative study. The values for the in vitro and in vivo samples were different. This may be because in in vivo experiment the entire plant is responding while in the in vitro experiment the leaf is disconnected from the plant and hence the response is different.

Both the varieties showed general resistance towards infection with *Alternariabrassicicola*. However, based on the present study it was found that Bio-902 was more resistant than the PCR-15. Some of the plants could not survive infection, may be due to heavy infection or time lag between elicitation of infection and resistance response established by the host.

Change in Salicylic Acid Content

Colorimetric estimation

Treatment of *Brassica campestris* with *Alternaria brassicicola* shows an increase in SA content which shows induction of SAR (Matreanx *et al*., 1990).

Methods employed for SA content measurement based on the colour formation reaction of ferric ions of colour reagents with phenolics groups of SA. The salicylic acid contents of 2 different hrs of plant at different hrs. of inoculation with pathogen in terms of mg/g fresh weight is depicted in table no. 1, 2, 3, and 4.

The observation shows a general trend of increase in SA content of all the two varieties than the normal ones. The finding is in the agreement with those of Wand *et al*., 1991.

Among the different age group of plants, it was seen that in in vitro experiment both 20 d and 25 d old plant give the best response to the infection with higher SA content than in vivo experiment.

In Bio-902 maximal increase in SA content was observed after 24 hrs. Of inoculation, this is more pronounced in in vitro experiment than in in vivo. In general, there was gradual increase in SA content up to 24 hrs. followed by gradual decrease up to 72 hrs. was observed in 20 d old plant. PCR-15 shows maximal SA content after 4 hrs. of infection in 20 d old plant, as well as 25 d old plant. On comparing SA content of the two varieties after infection, it can be seen that SA content of Bio-902 variety is higher than that of PCR-15.

SA is a SAR-inducing signal (Raskin, 1992) [11]. SA seems to be a mobile signal produced by the inoculated leaf which travels through the vascular tissue to uninoculated leaves where it induces the PR expression. This is well evidenced by the increase of SA amount in different variety after infection observation of the increase of the SA amount till 24 hrs is in agreement to Smith-Beaker *et al*., (1998).

Summary and Conclusion

The present study reveals the accumulation of Salicylic acid in the fungal elicited plants. The aim of present study was to compare the level of resistance in two varieties of rapeseed. Based on finding it was found that both the varieties showed resistance against infection with *A.brassicicola*. Bio-902 was found somewhat more resistant than PCR-15. Aforementioned facts, though present a biochemical aspect of the picture, in toto, still a lot is to be done from the point of view of genetic engineering i.e. to produce "disease resistant" transgenic plant varieties.

It is hoped that more useful and innovative research approaches to unravel the mystery of SAR and plant immunity will be focused in future and these aforementioned findings will be serve as a step in the series of steps of the ladder for that purpose.

Table 1: Quantitative changes in Salicylic Acid content in intact leaves (in vivo) of 25 d old plant of different varieties of *Brassica campestris* after infection with *Alternaria brassicola*.

Variety	Hrs. after inoculation	Salicylic Acid content mg/gfw		% change
		Normal	Infected	
BIO-902	0	7.14	7.21	0.98
	4	8.85	9.42	6.40
	24	5.64	7.78	37.94
	72	6.14	6.49	5.8
PCR-15	0	3.71	3.76	1.3
	4	4.80	6.28	30.8
	24	4.91	5.90	20.16
	72	6.22	6.76	8.68

Table 2: Quantitative changes in Salicylic acid content in excised leaves (in vitro) of 25 d old plant of different varieties of *Brassica campestris* after infection with *Alternaria brassicicola*.

Variety	Hrs. after inoculation	Salicylic Acid content mg/gfw		% change
		Normal	Infected	
BIO-902	0	6.92	6.99	1.01
	4	7.14	7.42	3.92
	24	8.92	12.43	39.34
	48	878	11.57	31.77
	72	8.86	9.50	7.22
PCR-15	0	3.10	3.10	0.00
	4	5.33	6.61	24.01
	24	6.15	6.97	13.33
	48	4.67	5.02	7.49
	72	5.12	5.23	2.14

Table 3: Quantitative changes in Salicylic acid content in excised leaves (in vitro) of 20 d old plant of different varieties of *Brassica campestris* after infection with *Alternaria brassicicola*.

Variety	Hrs. after inoculation	Salicylic Acid content mg/gfw		% change
		Normal	Infected	
BIO-902	0	6.74	6.74	0.00
	4	7.33	7.66	4.50
	24	7.91	9.16	15.80
	48	8.41	10.50	24.85
	72	8.33	9.16	9.96
PCR-15	0	4.94	4.99	1.2
	4	5.78	7.16	23.87
	24	6.05	6.83	12.91
	48	6.38	7.05	10.50
	72	6.70	7.11	6.11

Table 4: Quantitative changes in Salicylic acid content in intact leaves (in vivo) of 20 d old plant of different varieties of *Brassica campestris* after infection with *Alternaria brassicicola*.

Variety	Hrs. after inoculation	Salicylic Acid content mg/gfw		% change
		Normal	Infected	
BIO-902	0	6.92	6.99	1.01
	4	7.14	7.42	3.92
	24	8.92	12.43	39.34
	72	8.86	9.50	7.22
PCR-15	0	3.10	3.10	0.00
	4	5.33	6.61	24.01
	24	6.15	6.97	13.33
	48	4.67	5.02	7.49
	72	5.12	5.23	2.14

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