Secretion of cytokinins in vivo and in vitro by *Alternaria brassicicola* and their role in pathogenesis

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Summary. Formation of green islands in the host (mustard leaves) beneath the infection-drops containing germinating conidia of *Alternaria brassicicola* (Schw.) Wiltsch. has been correlated with the secretion of cytokinins by the pathogen. *A. brassicicola* also synthesized cytokinins in the liquid synthetic medium. Cytokinins produced in vitro were extracted, and their application on the detached mustard leaves evoked the formation of green islands.

It is well recognized now that cytokinins are involved in the following aspects of the diseases caused by biotrophs; green island formation, formation of metabolic sinks, abnormal nutrient transport from healthy to infected parts, and accumulation of these nutrients at infection sites[1-4]. However, much less information is available for the diseases caused by hemi-biotrophs. Mandahar and Arora[5,6], while working on the *Helminthosporium turcicum* – maize complex, found that green islands were present beneath the infection-drops containing germinating conidia. On this basis they hypothesized the possible involvement of cytokinins in pathogenesis by the hemi-biotroph. To find out whether the same phenomenon occurs in another hemi-biotroph, we conducted experiments on the *Alternaria brassicicola* – mustard complex.

Material and methods. The fungus was isolated from naturally infected mustard leaves by the single spore isolation method. No morphological variation was found in the various isolates of fungus. Only 1 of the representative monosporic isolates of the fungus was maintained.

For in vitro excretion of cytokinins, the fungus was cultured in synthetic medium of the following composition: glucose, 20 g; KH_2PO_4, 5 g; KNO_3, 5 g; $MgSO_4$. $7H_2O$, 500 mg; distilled water to make 1000 ml. Hydrogen ion concentration was adjusted to 6 with 1N NaOH. 40 ml aliquots of the medium were pipetted into each 100 ml conical flask. All flasks containing culture medium were sterilized at 15 lbs per square inch steam pressure for 20 min. The inoculum in each case consisted of 1 ml of spore suspension prepared in autoclaved distilled water, and contained 1×10^5 conidia. The culture flasks were incubated at 25 °C for 25 days. After incubation the mycelium was removed by filtration and culture filtrate was pooled to give a final volume of 2 l. This was condensed to 100 ml by evaporation under vacuum at 30 °C. Cytokinins were then extracted from it by the method of Kiraly et al.[1]

For in vivo study of cytokinins, infection-drops containing 1×10^5 conidia/ml of *A. brassicicola* were put on detached fully expanded uninjured healthy mustard leaves and kept in moist chambers in the dark. The spores germinated, produced appressoria and infection pegs and successfully established growth within the host within 20 h of inoculation. 4 h later, i.e. 24 h after inoculation, green islands were visible underneath the infection-drops. The leaves were kept in dark for 48 more hours, by which time the green islands had become very clear while the surrounding tissue had turned completely yellow. Then (i.e. after 72 h of inoculation) infection-drops were pipetted off and the green islands formed under infection-drops and the surrounding yellowed tissue (10 g of each) was treated to extract endogenous cytokinins.

10 g fresh weight of leaf tissue was ground in 10 ml of distilled water with pestle and mortar; the macerate was centrifuged at 3000 rpm for 5 min, and the supernatant was taken for extraction of cytokinins. Cytokinins were extracted from the supernatant as well as from the condensed culture filtrate by the method of Kiraly et al.[1] as given below. Both were adjusted to pH 2.9 by O.1N HCl and then extracted with 10 ml aliquots of ethyl ether. The aqueous phase was adjusted to pH 7.8 (by O.1N NaOH) and then extracted with three 20 ml aliquots of n-butanol. The butanolic fractions containing the active ingredient were combined and evaporated to dryness under vacuum at 30 °C. The residue left was dissolved in 30 ml of distilled water, in the case of cytokinin extract from fresh leaves, and 100 ml distilled water in the case of the cytokinin extract from culture filtrate.

The 3 biological assays performed for determination and estimation of cytokinins were root elongation inhibition test[1], radish cotyledonary leaf fresh weight increase and leaf expansion test[7], and chlorophyll retention test[8]. For quantitative estimation, the cytokinin-like activity of the test solution was compared with different concentrations of kinetin solution ranging from 10 to 150 µg/ml in distilled water. Sugar, starch and pro-

Table 1. Cytokinin-like activity of extracts obtained from the green islands (formed on mustard leaves) and from culture filtrate of *A. brassicicola*

Extract	Concentration	Bioassays: Barley root elongation inhibition		Radish cotyledonary leaf fresh weight increase and leaf expansion test				Chlorophyll retention test	
		Average root length (cm)	Percent inhibition as compared to blank	Average wt. of cotyledons (mg)	Percent increase in wt. as compared to blank	Average area of cotyledons (cm^2)	Percent increase in area as compared to blank	Amount of chlorophyll at 662 nm	Percent chlorophyll retained in excess as compared to blank
Green islands[a]	100	1.35 ± 0.16	79.54	23.01 ± 0.21	120.61	0.510 ± 0.005	128.69	0.075 ± 0.002	650
	50	2.65 ± 0.12	69.84	16.01 ± 0.13	53.49	0.368 ± 0.003	65.02	0.037 ± 0.001	270
	25	3.75 ± 0.11	43.18	13.30 ± 0.16	27.51	0.297 ± 0.002	33.18	0.023 ± 0.002	130
Tissue surrounding green islands	100	5.7 ± 0.23	13.63	10.71 ± 0.18	2.68	0.240 ± 0.001	7.62	0.01 ± 0.001	–
	50	6.3 ± 0.19	4.54	10.53 ± 0.12	0.958	0.232 ± 0.002	4.03	0.01 ± 0.002	–
	25	6.0 ± 0.22	9.09	10.43 ± 0.17	–	0.230 ± 0.001	3.13	0.01 ± 0.001	–
Blank	–	6.60 ± 1.04	–	10.43 ± 0.15	–	0.223 ± 0.001	–	0.01 ± 0.002	–
Culture filtrate[b]	100	0.62 ± 1.9	90.67	40.50 ± 0.01	288.42	0.881 ± 0.002	289.82	1.76 ± 0.001	17500
	50	4.22 ± 0.47	36.54	13.30 ± 0.03	27.81	0.289 ± 0.003	27.57	0.021 ± 0.003	110
	25	5.58 ± 0.6	16.09	11.50 ± 0.02	10.57	0.250 ± 0.001	10.61	0.013 ± 0.001	30
Blank	–	6.65 ± 1.04	–	10.40 ± 0.24	–	0.226 ± 0.001	–	0.01 ± 0.001	–

[a] After 72 h incubation in the dark at 30 °C; [b] after 25 days incubation at 30 °C.

Table 2. Percent increase in reducing sugar, starch and protein content in green islands[a] formed on mustard leaf as compared to the surrounding tissue

Nutrient	Green island	Surrounding tissue	Percent increase as compared to surrounding yellowed tissue
A. in green islands formed under infection-drops			
Reducing sugar (O.D. at 665 nm)	0.68	0.16	325.0
Starch (O.D. at 665 nm)	0.25	0.10	150.0
Protein (O.D. at 660 nm)	0.17	0.11	50.0
B. in green islands formed under drops of cytokinin extracted from culture filtrate			
Reducing sugar (O.D. at 665 nm)	1.50	0.16	837.5
Starch (O.D. at 665 nm)	0.34	0.10	240.0
Protein (O.D. at 660 nm)	0.19	0.10	90.0

[a] After 72 h incubation in dark at 30 °C.

tein were estimated by the methods of Yem and Willis[9] and Lowry et al.[10] respectively.

Results and discussions. Taking the original concentration of extract to be 100%, bioassays were performed with 100, 50 and 25% concentrations of extract obtained from green islands/yellowed tissue/culture filtrate. The results indicated that there was 79.54, 59.84, 43.18% inhibition in root length of barley seedlings over blank; 120.61, 53.49, 27.51% increase in fresh weight of cotyledons over blank; 128.69, 65.02, 33.18% increase in area of radish cotyledons over blank; and 650, 270, 130% chlorophyll retention in cotyledons over blank when different dilutions of cytokinins, extracted from green islands formed on leaf tissue under infection drops containing germinating conidia of *A. brassicicola*, were bioassayed. When results of the above tests were compared with the calibration curve the concentration of the active substance in the green island extract was found to be equivalent to 47 µg/ml of kinetin. Except for a little inhibition in root length of barley seedlings, the results of other bioassays were the same as in the blank when bioassays were performed with cytokinins extracted from yellowed tissue.

When cytokinins extracted from culture filtrate of *A. brassicicola* were bioassayed, 90.67, 36.54, 16.04% inhibition in root length of barley seedlings was obtained over blank; 289.42, 27.81, 10.37% increase was obtained in fresh weight of radish cotyledons over blank; 289.82, 27.87, 10.61% increase in area of radish cotyledons was obtained over blank; and 17500, 110, 30% chlorophyll retention in cotyledons over blank was observed. When results of the above tests were compared with the calibration curve, concentration of the active substance in the extract was found to be equivalent to approximately 119 µg/ml of kinetin.

All bioassays show the presence of cytokinins in the green islands formed on detached mustard leaves at areas beneath the infection-drops containing germinating conidia of *A. brassicicola*. Since the leaves had been detached, it is obvious that cytokinins in the green islands must have been secreted by the pathogen. This is confirmed by the secretion of cytokinins by the fungus in liquid synthetic medium. Moreover, treatment of mustard leaf (healthy, detached) with cytokinins extracted from the culture filtrate of *A. brassicicola* evoked the formation of green islands after 72 h incubation in dark.

Since cytokinins delay senescence in excised leaves[11-13], it appears that the cytokinins secreted by the germinating conidia in the infection-drops and in the culture medium lead to the retardation of senescence in the leaf tissue. It is clear from all that has been discussed above that cytokinins secreted by the germinating conidia of *A. brassicicola*, like those from *Helminthosporium turcicum*, may create localized translocatory sinks at quite an early stage. The nutrients move towards these sinks from the surrounding areas and supply food and energy to the growing pathogen for penetration and for further development after penetration.

This was confirmed by actual quantitative estimation of sugar, starch and protein content in green islands. The sugar, starch and protein contents were 325, 150, 50% more in green islands formed beneath the infection-drops of *A. brassicicola* than in the surrounding tissue. In green islands formed under the drops of cytokinin extracted from culture filtrate of *A. brassicicola* sugar, starch and protein contents were 837.5, 240, and 90% respectively more than in the surrounding tissue (table 2).

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The elimination of 1,5-anhydroglucitol administered to rats

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Summary. Rat serum contains natural 1,5-anhydroglucitol. Injected or orally administered 1,5-anhydroglucitol was efficiently reabsorbed by the renal tubuli via a mechanism which had a saturation point at high serum 1,5-anhydroglucitol levels. The compound had a slow turnover rate in the body; its half-life is approximately 3 days. The compound was readily absorbed in the gut when administered orally.

1,5-anhydroglucitol (AG) is one of the major polyols of human cerebrospinal fluid[1,2] and plasma[3-5]. AG is a nonreducible C_6-monosaccharide which appears to be freely filterable through the renal glomeruli. Studies in humans have suggested that the renal tubular cells reabsorb AG, since no AG has been found in the urine[5]. An association has been discovered between