Mycobiology 32(4): 155-159 (2004)

Effect of the Mixture of Two Plant Alkaloids Isolated from *Corydalis longipes* Against Balsam Powdery Mildew on Detached Leaves and Pea Powdery Mildew in Field

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(Received October 28, 2003)

N-Methylhydrasteine hydroxylactam and 1-methoxyberberine chloride, both alkaloids, extracted from *Corydalis longipes* have been assayed for their activities against two powdery mildews. The spore germination of *Erysiphe cichoracearum* on detached leaf of balsam (*Impatiens balsaminia*) following pre- and post-inoculation treatments by their mixture has shown high efficacy against the pathogen at 100, 200 and 300 μg/ml. The mixture was also effective at both pre- and post-inoculation treatments at 500, 1000, 1500 μg/ml doses against *E. pisi* causing pea powdery mildew in pea (*Pisum sativum*) under field conditions. The significant efficacy of the mixture of two compounds against spore germination on detached leaves of balsam and also under field conditions in pea warrants its inclusion in trials against some other diseases under field conditions.

KEYWORDS: ***Corydalis longipes*, *Erysiphe cichoracearum*, *Erysiphe pisi*, 1-Methoxyberberine chloride, N-Methylhydrasteine hydroxylactam, Spore germination**

There has always been a need of increased agricultural production but one of the major constraints faced is of fungal diseases. To overcome this problem, use of synthetic fungicides came into being with satisfactory results. However, with continuous use of such fungicides a number of ecological and health problems were also realized in terms of resistance among various fungi, human health and ecological imbalances. This drew the attention of researchers for developing eco-friendly methods to control fungal diseases such as genetic engineering for developing resistant varieties and use of induced resistance by biotic and abiotic means (Lyon *et al.*, 1995), biocontrol agent (Vidyasekaran and Muthamilan, 1995) induced systemic resistance and by natural products (Prithiviraj *et al.*, 1996, 1997; Singh *et al.*, 1980, 1988). Plants contain a large number of anti-fungal compounds (Pan *et al.*, 1985) and many of them, particularly phenolics, have been implicated in natural resistance of several plants against pathogens. The plant extracts/products have shown promising results against phytopathogenic fungi *in vitro* (Basha *et al.*, 2002; Chaturvedi *et al.*, 1987; Khurana *et al.*, 1972; Maillard *et al.*, 1998; Maurya *et al.*, 2002) and also under field conditions (Prithiviraj *et al.*, 1998; Sriobaite *et al.*, 1960).

The successful results of the plant extracts/products, both *in vitro* and *in vivo*, against plant pathogens led to find out of the possible anti-fungal effect of the mixture of N-methylhydrasteine hydroxylactam and 1-Methoxyberberine chloride in the ratio of 1 : 1 extracted from *Corydalis longipes*, both *in vitro* and *in vivo* conditions and the results are presented here.

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Materials and Methods

Extraction of plant alkaloids. *Corydalis longipes* D.C.Prodr. (Fumariaceae) grows on shady moist places at 2290~2350 m altitude and distributed from Garwal hills to Sikkim in Himalayas and throughout Nepal. A number of medicinal values have been reported in Indian and Chinese system of medicines for *Corydalis* (CSIR, 1950) species.

The dried powder of the whole plant of *C. longipes* (1.5 kg) was extracted with methanol in a soxlet apparatus. After removal of solvent, the methanol extract was extracted with citric acid (7%) by stirring mechanically for four hours and then filtered. The acidic filtrate was basified with ammonium hydroxide and extracted further with chloroform several times vigorously by shaking in a separating funnel till the acidic solution gave no test for alkaloids. The combined chloroform extract was evaporated to dryness, which gave crude alkaloidal fraction as brown semi-solid. The mixture of crude bases was then chromatographed over silica gel column eluting with solvents of increasing polarity and all eluted fractions were monitored by thin layer chromatography for their homogeneity. The identical eluants were mixed together and crystallized from methanol. The $CH+Cl_3$-MeOH (95 : 5) and (9 : 1) eluants furnished alkaloids, N-methylhydrasteine hydroxylactam and 1-methoxyberberine chloride. These compounds were identified by spectral studies as mentioned below.

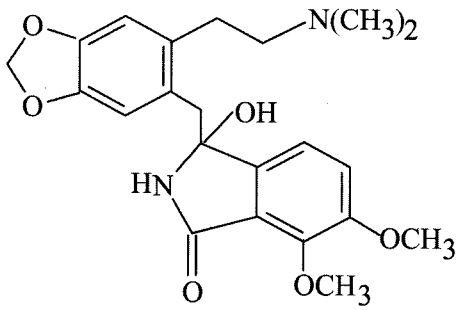

Fig. 1. Structural formula of N-methylhydrasteine hydroxylactam.

N-Methylhydrasteine hydroxylactam: It crystallized as colourless needles, mp.110~113°C and showed light orange colour with Dragendroff's reagent indicative of alkaloids. The molecular formula was settled as $C_{22}H_{26}N_2O_6$ from its high resolution mass spectra. It exhibited UVλ max (MeOH) : 216, 293, 315 nm and IR ζ max (KBr): 3220 (NH), 1750 (lactam) cm^{-1}. The above data together with chemical shifts in[1] HNMR and [13]CNMR were identical with the reported data of N-methylhydrasteine hydroxylactam (Fig. 1) (Blasko *et al.*, 1972). The identity of the compound was further proved by direct comparison with authentic sample (mixed mp., co-TLC and superimposable IR).

1-Methoxyberberine chloride: It crystallized as yellowish powder, mp.166~168°C and gave light orange colour with Dragendorff's reagent which indicated it to be an alkaloid. The molecular formula was determined as $C_{22}H_{20}O_5$ (M+366) from mass spectrum. It exhibited UVλ max (MeOH): 234, 262, 282, 330, 342, 428 cm^{-1}. The above data together with chemical shifts in HNMR were identical to the reported data of 1-methoxyberberine chloride (Fig. 2) (Rücker *et al.*, 1994). It was further identified by direct comparison with authentic sample (mixed m.p.; co-TLC and super imposable IR). The alkaloids, N-methylhydrasteine hydroxylactum (Fig. 1) and 1-methoxyberberine chloride (Fig. 2) were mixed in equal proportion and subjected to anti-fungal activity.

Effect of the mixture of compounds on powdery mildew (*Erysiphe cichoracearum*) development on detached leaves of balsam (*Impatiens balsaminia*). Second nodal leaves from 20-day-old balsam (*Impatiens balsaminia*) plants were excised with the help of sharp scissors and spread on moist filter papers. The leaves were then inoculated by *Erysiphe cichoracearum* conidia on adaxial surface by tapping heavily infected leaves so as to get 200~300 spores per mm^2 of the leaf area. These leaves were then floated on sterilized distilled water in Petri plates, which served as control. Different dilutions of the chemical (100, 200 and 300 $\mu g/ml$) were sprayed on detached pea leaves thoroughly with the help of a hand atomizer 24 h prior to seeding the excised leaves. These leaves were later floated on distilled water in Petri plates keeping adaxial surface up. After 24 h of spraying, the leaves were then seeded with *E. cichoracearum* conidia by tapping heavily infected leaves. All the plants were incubated at 25±2°C. For post-inoculation treatment, the experiments were conducted as stated above except that the spores were tapped first and after 24 h the chemical was sprayed by a hand atomizer on the seeded leaves, which were then again floated on distilled water in Petri plates and later incubated at the same temperature (25±2°C) as described above.

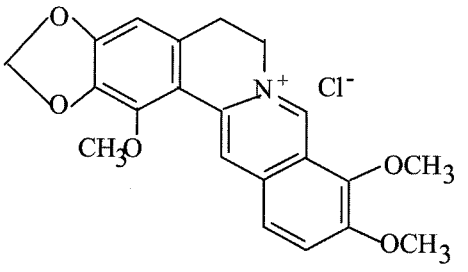

Fig. 2. Structural formula of 1-methoxyberberine chloride.

After incubation for 24 h the leaves were fixed and stained by the method of Carver and Adaigbe (1990). A pad of filter paper was placed in a Petri plate containing the fixative (Ethyl alcohol-acetic acid, 3 : 1). The leaves were placed on the filter paper with adaxial side up to minimize the disturbance of conidia on the leaf surface. The leaves were fixed for 48 h to remove the chlorophyll completely. They were then placed on filter paper pads soaked with lactophenol for another 24 h to soften the leaf tissue, and mounted in lactophenol-cotton blue for staining the conidia. Observations regarding unipolar and bipolar germination, number of germ tubes and number of appressoria were made under light microscope. All the experiments were conducted in triplicate and subjected to randomized block design for statistical analysis.

Field efficacy of the mixture of compounds on powdery mildew (*Erysiphe pisi*) of pea (*Pisum sativum*). The field was properly ploughed, followed by leveling in order to conserve moisture for proper seed germination. The field was divided into several plots and each plot was of 2×2 m in size. The seeds were sown in drills in line, maintaining line to line distance of 30~45 cm and seed to seed spacing of 5~7.5 cm at 2.5~5 cm depth. The seeds were sown at the rate of 60~100 kg per hectare. The efficacy of the chemical was evaluated in the field on 20~25 day-old plants. The plants received pre- and post-treatments of the chemicals. During pre-inoculation, chemical was sprayed with the help of a hand atomizer on the selected plants. After 24 h the plants were inoculated with *Erysiphe pisi* conidia by tapping heavily infected leaves on the adaxial surface of the leaves. For post-inoculation treatment, the plants were inoculated first with the conidia

of *Erysiphe pisi* on adaxial surface of the leaf. After 24 h the chemicals were sprayed on the surface of the leaf using a hand atomizer with different concentrations. The control plants received only the inoculum. The plants were left and observed till lesion development. All the experiments were done in triplicate. Disease intensity was recorded at 3 days interval till 15 days of the treatments by using the following formula:

$$\text{Disease intensity (\%)} = \frac{\text{Sum of rating (0–4 scale)}}{\text{Maximum possible score} \times \text{No. of leaves observed}} \times 100.$$

The rating was done as:
0 = No powdery mildew symptoms
1 = 5% leaf area infected
2 = 18% leaf area infected
3 = 38% leaf area infected
4 = 75% leaf area infected.

Results and Discussion

Effect of mixture of the two compounds was seen on detached leaves of balsam against pea powdery mildew (*E. cichoracearum*) in Petri dishes containing water as floating medium was seen. The results showed that the pre- and post- inoculation treatments with different concentrations (100, 200, 300 *μg/ml*) had significant inhibitory effect on spore germination following inoculation on leaves of balsam. The result of individual treatment indicated that the post-inoculation treatment was better than the pre-inoculation as percent spore germination was less than 20% in post-inoculation while that in pre-inoculation was about 30% at 300 *μg/ml* (Fig. 3).

The effect of the mixture was also seen against pea powdery mildew (*E. pisi*) under field conditions. Though both the treatments affected the development of powdery

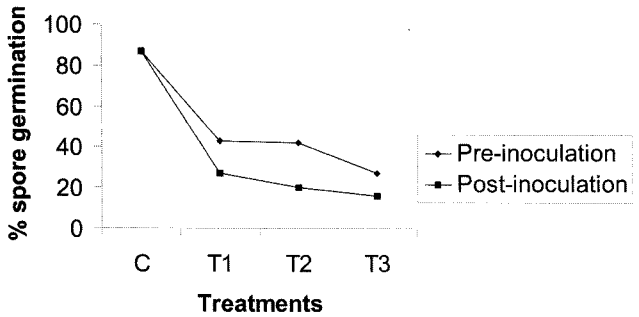

Fig. 3. Effect of pre- and post-inoculations of N-methylhydrasteine hydroxylactam and 1-methoxyberberine chloride on lesion development of powdery mildew of balsam (*Impatiens balsaminia*) *in vitro* (T1=100 *μg/ml*, T2=200 *μg/ml*, T3=300 *μg/ml*).

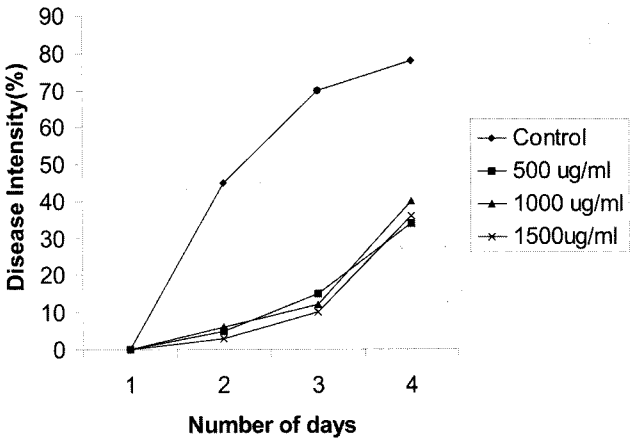

Fig. 4. Effect of pre- inoculation treatment with mixture of N-methylhydrasteine hydroxylactam and 1-methoxyberberine chloride on disease intensity of powdery mildew of pea (*Pisum sativum*) in field (Data taken after an interval of 7 days).

mildew on pea plants, the post-inoculation treatment was more effective then pre-inoculation at 1500 *μg/ml* in reducing the disease intensity by about 20%. However, all the three concentrations (500, 1000, 1500 *μg/ml*) in the field were significantly effective in both the treatments in reducing disease intensity as compared to control (Figs. 4, 5).

The effect of mixture of compounds on detached leaves of balsam powdery mildew *in vitro* and pea powdery mildew under field conditions revealed that the mixture was effective against both the biotrophs at very low concentration. While spore germination of *E. cichoracearum* was significantly inhibited on detached leaves of balsam at 500 *μg/ml* under control conditions, the same mixture was

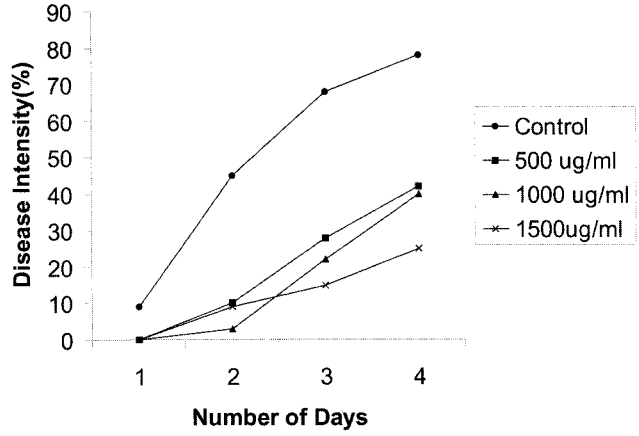

Fig. 5. Effect of post-inoculation treatment with mixture of N-methylhydrasteine hydroxylactam and 1-methoxyberberine chloride on disease intensity of powdery mildew of pea (*Pisum sativum*) in field (Data taken after an interval of 7 days).

Table 1. Effect of plant alkaloids on spore germination of some fungi

Fungus	Host	Percent spore germination							C.D. at 1%
		Concentration in μg/ml							
		C	C+M	50	100	150	200	250	
Alternaria alternata	*Rhaphanus sativus*	98.67	99	91.67**	88.3**	76.67**	76.67**	0.67**	4.79
Alternaria brassicae	*Brassica campestris*	99	98.67	91**	87.67**	83.67**	75.67**	0	4.40
Colletotrichum gloeosporioides	*Mangifera indica*	97.67	96.67	57.67**	55.3**	27**	25**	13.67**	8.19
Curvularia pallescans	*Bambusa indica*	98.67	92.67	0	0	0	0	0	4.41
Curvularia maculans	*Musa paradisiaca*	93.3	93	17.67**	5**	0	0	0	9.3
Fusarium udum	*Cajanus cajan*	99.67	99.3	46**	0	0	0	0	5.8
Fusarium sp.	*Albizza lebbek*	98.3	96.3	87**	1.3**	0	0	0	3.42
Helminthosporium echinochlova	*Echinocloa* sp.	99	99.67	25**	26**	21.6**	22.67**	0.67**	9.08
Helminthosporium speciferum	*Solanum melongena*	99	98	55.3**	45.3**	21.3**	14**	0	15.9
Ustilago cynodontis	*Cynodon dactylon*	91	92.3	31.67**	13.67**	0	0	0	9.46

**-Data is significantly different from control, C=Control, C+M=Control+methanol, C.D.=Critical Difference.

highly effective at slightly higher concentration (1500 μg/ml) on pea powdery mildew under field conditions. The higher concentration required in the field is because of the fact that the chemical had to interact with a number of environmental conditions. However, this concentration (1500 μg/ml) is well within the recommended dose of commercial fungicides used by farmers to control plant diseases including powder mildews. Seeing the results on detached leaves of balsam against *E. cichoracearum* and under field conditions against pea powdery mildew it can be inferred that the chemical is highly effective against the two different powdery mildews. It can, therefore, be concluded that the mixture can be recommended for use by farmers for powdery mildews under field conditions. The chemical can also be tried against some other disease *in vivo* for its efficacy. Since both the compounds are of plant origin it is expected that they may take care of biosafety and environmental hazards.

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