

**Table 1.** Growth performance of two aconite species under two environmental conditions in alpine environments

Species	Parameters	Growth environment	
		Nature	Polyhouse
<i>Aconitum heterophyllum</i>	Plant height (cm)	40.80 ± 5.45	123.40 ± 21.77
	No. of leaves per plant	13.80 ± 2.17	19.60 ± 3.51
	No. of flowers per plant	17.00 ± 4.00	45.20 ± 17.05
	No. of new tubers per plant	1	1
	Av. length of tubers per plant	4.5	13.0
	Dry wt of tubers (g) per plant	1.10	12.10
	Per cent alkaloids (g per 100 g dry wt)	1.82	2.23
<i>Aconitum balfourii</i>	Plant height (cm)	120.20 ± 5.12	203.20 ± 32.03
	No. of leaves per plant	49.00 ± 3.39	58.80 ± 16.53
	No. of flowers per plant	64.00 ± 6.04	61.00 ± 28.33
	No. of new tubers per plant	1	5
	Av. length of tubers per plant	7.00	1.20
	Dry wt of tubers (g) per plant	8.76	69.00
	Per cent alkaloids (g per 100 g dry wt)	1.72	1.99

The alkaloidal fraction (0.01 mg) was dissolved in HPCL mobile phase (hexane : chloroform : methanol; 70 : 20 : 10) and filtered through 0.45 millipore filter. Normal phase silica-18 column (4.5 × 250 mm) with the same mobile phase at a flow rate of 1 ml/min was used to work out the retention behaviour of aconitine and atisine by injecting 20 µl samples. The standard alkaloids were detected  $\lambda_{\max}$  240 nm. Contents were calculated by the standard peak-area method. It can be seen that the quantity of aconitine as well as atisine was higher in plants grown under polyhouse conditions.

These results clearly indicate that the present market demand can be met by cultivating these species in polyhouses. The cost of cultivation would be around Rs 292,000 per acre and the production would be 8 to 11-fold higher than under open field conditions where the cost of cultivation would be about Rs 41,800 per

acre. Keeping in view the present market rates, the net profit in polyhouse cultivation would be Rs 195,000 per acre and Rs 348,000 per acre in *A. balfourii* and *A. heterophyllum*, respectively. Whereas the cultivation in open fields would give a net profit of only Rs 20,300 and Rs 17,000 per acre in these plants. Cultivation following such methods will reduce the extraction of these plants from nature leading to their conservation in nature. Such a method of cultivation could be followed even in temperate conditions.

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M. C. NAUTIYAL  
A. N. PUROHIT\*

High Altitude Plant Physiology Research Centre,  
H.N.B. Garhwal University,  
Srinagar Garhwal 246 174, India  
\*For correspondence.  
(e-mail: happrc@nde.vsnl.net.in)

## Antagonistic potential of N<sub>2</sub>-fixing *Acetobacter diazotrophicus* against *Colletotrichum falcatum* Went., a causal organism of red-rot of sugarcane

Use of biological control agents present in nature has been widely studied for new eco-friendly approaches to control pests and pathogens. Application of beneficial bacteria as agents for biological control of plant diseases in agriculture is an emerging potential alternative to chemical fungicides. Some bacteria like *Enterobacter cloacae* protect the economically

important plants against *Pythium* pre-emergence damping off caused by binding of *P. ultimum* to fungal hyphae<sup>1</sup>.

*Bacillus thuringiensis* is reported to control many insects by producing different chemical compounds<sup>2</sup>. *Pseudomonas putida* reduced sheath blight incidence by 18% in comparison with infected control<sup>3</sup>. Evidences for mechanisms of

biological control of pathogens can be exemplified by many factors that include competition, production of antibiotics, siderophores, etc. Endophytic pseudomonads surviving in different host plants hold the potential for the suppression of rice diseases<sup>4,5</sup>.

Sugarcane crop is prone to diseases when climatic shifts are rapid. The oldest

known red-rot disease of sugarcane caused by *Colletotrichum falcatum* Went., occurs in most of the countries where sugarcane is being cultivated. The dreadful disease was a cause for the failure of many good varieties of sugarcane and is still a major threat in subtropical areas causing sett rot. *In vitro* production of toxic metabolites by *C. falcatum* has been demonstrated<sup>6</sup> and the toxin produced by this fungus at a concentration of 0.5% and above inhibited the callus growth of sugarcane<sup>7</sup>. The primary infection of this disease is through infected setts and the secondary infection is from the soil, water and wind besides the sporulating masses at the nodes affecting the adjacent standing canes. When secondary infection occurs in the standing crop, control measures such as fungicidal or any bio-cidal applications cannot hold promise as containment of the disease becomes very difficult due to its mode of infection at all stages of growth. One possible method to control this disease can be developing an in-built mechanism either through gene manipulation or alteration in cane physiology as the disease occurs in resistant varieties also. Another method may be through microbes that can be associated in the entire plant system throughout the period of plant growth, having the potential of antagonism towards the pathogen. The role of diazotrophic bacteria has been envisaged as antagonistic against plant pathogens<sup>8</sup>. *Acetobacter diazotrophicus* is reported to exist in the entire plant system of sugarcane<sup>9,10</sup>. This study has been initiated to test the effect of *A. diazotrophicus* against the red-rot pathogen, *C. falcatum*.

For the isolation of *C. falcatum*, stem bits scrapped from red-rot affected sugarcane were initially cut into small pieces, washed with sterile water and sterilized with 70% alcohol (for 30 sec) followed by repeated washings in sterile distilled water. These were again sterilized with 0.1% HgCl<sub>2</sub> for 3 min, subsequently rinsed in sterilized double distilled water and placed on potato dextrose agar (PDA) in petri plates for incubation at 32°C. The fungal mycelia obtained after purification were identified using cultural and morphological characters. Identification was confirmed at the Centre of Advanced Study in Botany, University of Madras, Chennai. The fungal mycelia showing cottony growth turned grey and further to dark grey after 10 days. Under the micro-

scope, the hyphae were thin, branched, hyaline and septate. Numerous falcate, sickle-shaped conidiospores on unbranched conidiophores along with setae confirmed its identity.

Strains of *A. diazotrophicus* (the acetic acid producing, diazotrophic endophytic gram-negative bacterium) were isolated from different sugarcane varieties in Tamil Nadu<sup>10</sup>. The isolation was carried out by growing the cultures in LGI semi-solid medium (0.4% agar) in 10 ml serum vials containing 5 ml of the medium. Yeast extract (20 mg l<sup>-1</sup>) and cane sugar as a carbon source were supplemented. The isolates were identified as reported earlier<sup>9,10</sup>. To test the efficacy of the diazotrophic isolates on inhibiting the growth of red-rot pathogen, sterile PDA plates were seeded with mycelial discs (8 mm) of actively growing *C. falcatum*. The discs were placed at four corners, equidistant from the centre. Each test isolate of *A. diazotrophicus* was grown in LGI P liquid medium<sup>10</sup> for 24 h and a loopful was placed at the centre. The plates were incubated at 32°C for 7 days.

The strains which effectively suppressed the mycelial growth of *C. falcatum* were used for further studies.

Cultures of both the fungal pathogen and antagonistic diazotrophs (different strains) were grown in potato dextrose broth (PDB) with initial pH of 6.0. Actively growing *C. falcatum* on PDA medium was used for inoculation (8 mm discs) in order to determine the efficacy of these diazotrophic strains. The mycelial weight of the pathogen was recorded after 15 days by retaining the fungal mat when filtered through Whatman filter paper. The effective representative isolate of *A. diazotrophicus* was grown in 1 l of LGI<sup>9</sup> medium for 15 days, centrifuged (13000 rpm for 15 min at 4°C) and the supernatant obtained was filtered through 0.45 mm pore size millipore filter and reduced to one-tenth of the original volume by evaporating at 100°C in a water bath. This reduced volume was mixed with an equal volume of methanol, stirred well and kept at 4°C overnight and then filtered. After removing the methanol by distillation, the left out filtrate was taken into a separating funnel and the pH was adjusted to 3.5 using dilute 0.1 N HCl. An equal volume of diethyl ether was added and shaken well. The ether phase was then separated and mixed with an equal volume of 5% aqueous solution of

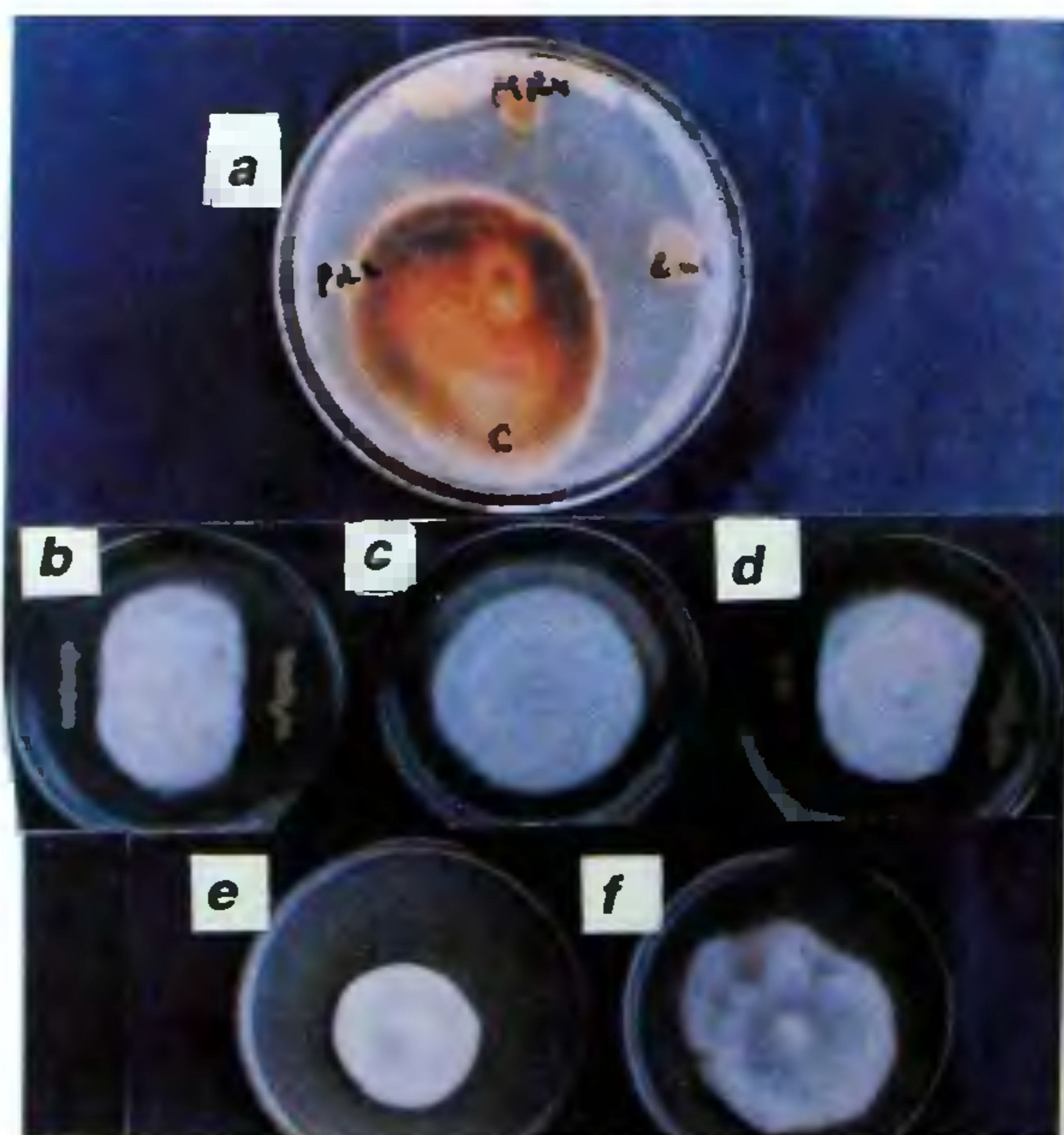


Figure 1. a, Screening of *A. diazotrophicus* against *C. falcatum*; b, Isolate AD 1 against *C. falcatum*; c, Control; d, Isolate AD 3 against *C. falcatum*. Growth of *C. falcatum* on PDA with 10 µg ml<sup>-1</sup> ECM (e) and without ECM (f) after 7 days.

sodium carbonate, separated again and the aqueous phase was discarded. The ether solution was evaporated to dryness at 40°C in a water bath for obtaining the extra cellular metabolites (ECM) and then weighed and kept in a desiccator for further use<sup>7</sup>.

Among 15 diazotrophic isolates of *A. diazotrophicus* screened in this study<sup>10</sup> for their inhibitory effect against *C. falcatum*, only 3 isolates showed positive result (Figure 1 a, b and d). A clear zone of inhibition occurred between *A. diazotrophicus* and *C. falcatum* suggesting diffusion of an antifungal substance from *A. diazotrophicus* through the PDA medium. The other possibilities are pH changes causing inhibition and depletion of essential nutrients as reported by Bull *et al.*<sup>11</sup>. Three isolates of *A. diazotrophicus* which exhibited marked inhibition were further used for a dual inoculation experiment using PDB. The result suggested that the test isolates of *A. diazotrophicus* (Table 1) exhibited 23 to 33% of growth inhibition of *C. falcatum* after 7 days. The initial pH of the medium (6.0) was reduced to 3.5 due to fermentation of sugars by *A. diazotrophicus*. However, *C. falcatum* when grown as monoculture did not show any pH reduction. It was reported that *A. diazotrophicus* ferments the sugars and reduces the pH of the medium below 3.0 (ref. 9) which may be the reason for growth inhibition of *C. falcatum*. Sterile ECM was aseptically removed through a membrane filter (0.4 µm pore size, Sartorius) from the growth medium of *A. diazotrophicus* and was tested *in vitro* against *C. falcatum*. Varying concentrations of the ECM (5, 10, 15 µg ml<sup>-1</sup>) were added to the PDB after sterilization. The result suggested that 10 µg ml<sup>-1</sup> of ECM could inhibit the growth of *C. falcatum* by 12.41% (Table 2, Figure 1 e). Growth inhibition of mycelia by ECM is less when compared to the dual inoculation in liquid medium in which acidification of the medium along with ECM may have affected the growth of the pathogen. The inhibition of the pathogen by *A. diazotrophicus* may be either due to its fungistatic or fungicidal property. Further studies on the identification of antifungal substances

**Table 1.** Antifungal activity of N<sub>2</sub>-fixing *A. diazotrophicus* on the growth of *C. falcatum* after 7 days in potato dextrose broth

Treatment	Mycelial wt.* (in mg)	Mycelial inhibition (%)
<i>C. falcatum</i> + AD 1	146.50	31.05
<i>C. falcatum</i> + AD 2	141.60	33.27
<i>C. falcatum</i> + AD 3	163.33	23.03
<i>C. falcatum</i> alone	212.20	0.00
Bavistin** (Carbendazym)	0.00	100.00
CD (at <i>P</i> = 0.05%)	0.34	—

\*, Mean of six replicates; \*\*, 10 ppm; AD 1, *Acetobacter diazotrophicus* (LMG – Type strain ATCC 49037<sup>T</sup>); AD 2 (MR4) and AD 3 (PS1), Isolates from India.

**Table 2.** Growth and mycelial inhibition of *C. falcatum* on potato dextrose broth by the ECM from *A. diazotrophicus*

Concentration of ECM (µg ml <sup>-1</sup> )	Growth of pathogen*	Mycelial inhibition (%)
Control	1.507	0.00
5	1.415	6.11
10	1.320	12.41
15	1.322	12.28
CD at ( <i>P</i> = 0.05%)	0.04	—

\*, Growth diameter of fungal mat in mm after 7 days, mean of 5 replicates.

will unravel the possible roles<sup>8</sup> of such diazotrophs apart from N<sub>2</sub>-fixation.

The *in vitro* test can give an indication as to what the results will be *in vivo*. The results obtained on agar and liquid media *in vitro* do not necessarily mean that the antagonist should be effective in field also<sup>12</sup>. However, the present study suggests the possibility of containing *C. falcatum* in future as *A. diazotrophicus* may provide an in-built mechanism within the sugarcane plant system. This can act against the pathogen due to its occurrence and colonization within the plant system throughout the season. The results also indicate that agronomically important *A. diazotrophicus* can also be used for biological control of red-rot disease-causing pathogen, apart from N<sub>2</sub>-fixation. Investigations with susceptible varieties of micropropagated sugarcane seedlings are in progress.

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R. MUTHUKUMARASAMY\*  
G. REVATHI  
M. VADIVELU

Main Bio-control Research Laboratory,  
(Unit of Tamil Nadu Co-operative Sugar  
Federation),  
2E/1 Rajeswari Vedachalam Street,  
Chengalpattu 603 001, India  
\*For correspondence.  
(e-mail: tncsf@md4.vsnl.net.in)