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Microbial antagonism to *Neovossia indica*, causing Karnal bunt of wheat[†]

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The potentiality of fungal, bacterial and actinomycete biocontrol agents isolated from soil was investigated against *Neovossia indica*. Culture filtrates of *Trichoderma pseudokoningii, T. lignorum, T. koningii, Gliocladium roseum, G. deliquescens, G. virens*, fluorescent pseudomonad isolate (FP-VII), bacterial isolate Bact-II and an actinomycete isolate act-V, inhibited germination of teliospore and secondary sporidia of Karnal bunt fungus. Bioassay test of the chloroform extracts of antagonists spotted on TLC plates indicated the presence of bands of different Rf values. Three bands showing involvement of atleast three antifungal compounds in case of *T. lignorum, T. koningii, T. pseudokoningii*, FP isolate (FP-VII), Bact-II and actinomycete isolate Act-V were observed. Bioassay of one of the purified compounds of *T. koningii* and *T. pseudokoningii* inhibited the mycelial growth of *N. indica* at 1000 and 500 ppm respectively while the compound from *T. lignorum* showed inhibition at 100 ppm.

KARNAL bunt of wheat caused by *Neovossia indica* (Mitra) Mundkur (*Tilletia indica* (Mitra)) not only causes reduction in yield but adversely affects the grain quality[1]. The disease is difficult to manage due to its complex nature. Although fungicide sprays are effective[2], their extensive use in controlling the disease is not environmentally safe and also not economical. Therefore, attempts have been made to prevent this disease by eco-friendly methods. In a study some antagonists like *Trichoderma viride, T. harzianum* and *G. deliquescens* have shown biocontrol potential[3], but the present investigations were undertaken to search for antagonistic micro-organisms with more potential against *N. indica*.

Fungal, bacterial and actinomycete cultures were isolated on selective media from soil samples of wheat rhizosphere collected from various locations and maintained on specific media[4,5].

Potato–dextrose poured petriplates inoculated with sporidial suspension (10^4 sporidia/ml) and seeded in the centre with the test organism were incubated at $18 \pm 1°C$ and observations on inhibition zone, over growth and lysis were recorded when colony growth of *N. indica* became visible. Petriplates inoculated with *N. indica* without any test organism served as control.

The selected fungal biocontrol agents were grown on potato–dextrose broth, bacteria and actinomycetes in nutrient broth in 100 ml conical flasks containing 40 ml media. The flasks were incubated at 25°C for 20 days in case of fungal cultures and 7 days for bacteria and actinomycetes. Cell free extracts were stored in McCartney's bottles at low temperatures (4°C) for undertaking further studies on antagonism.

A drop of culture filtrate of each antagonist was mixed with a drop of spore suspension (10^3 teliospores/ml and 10^4 sporidia/ml) on sterilized cavity slides and incubated at 18°C in moist chambers. The observations on percent teliospore and sporidia germination were recorded after 15 days and 4 h respectively.

About 1 litre cell free culture filtrate of each selected antagonist was raised and antifungal compound was ex-

tracted by solvent extraction method using chloroform. The solvent extracted portion was vacuum dried to obtain crude extract (oily residue) and stored in small glass vials for further studies. Bioassay with crude extract was done by agar well method. The most effective oily residues were further purified by thin layer chromatography (TLC).

The oily residues were spotted on TLC plates, having 0.25 mm thickness of silica gel layers. The plates were developed in hexane:benzene (1:1), air dried for 5 min and visualized by iodine. The spots were marked on the plates and Rf values were determined. For purification of antifungal compounds, oily residues of three most effective antagonists, viz., T. koningii (Tk), T. lignorum (Tl) and T. pseudokoningii (Tpk) were separated by column chromatography using a column (75 × 2 cm id) containing 50 g of 60–100 mesh silica gel (preactivated at 120°C). The column was successively eluted with hexane, hexane–ethyl acetate collecting 25 ml fractions which were distilled on a water bath and their purity checked on TLC plates.

Bioassay with 1000, 500 and 100 ppm of purified compounds was done by agar well method.

Analysis of the soil samples yielded 16 fungal cultures including Aspergillus terreus, A. nidulans, A. niger, A. flavus, A. flaviopsis, Aspergillus sp., Trichoderma koningii, T. hamatum, T. harzianum, T. lignorum, T. pseudokoningii, T. viride, Gliocladium roseum, G. penicilloides, G. deliquescens and G. virens.

Table 1. Effect of some micro-organisms on growth of Neovossia indica

Isolate	Inhibition zone (IZ) mm	Overgrowth/ lysis
Aspergillus niger (Ang-II)	5	–
Trichoderma koningii (Tk)	–	overgrowth & lysis
T. hamatum (Th)	–	over growth
T. harzianum (Tha)	–	over growth
T. lignorum (Tl)	–	over growth & lysis
T. pseudokoningii (Tpk)	–	over growth & lysis
T. viride (Tv-I)	–	over growth
(Tv-II)	–	over growth
(Tv-III)	–	over growth
(Tv-IV)	–	over growth
(Tv-V)	–	over growth
(Tv-VI)	–	over growth
Gliocladium deliquescens (Gd)	–	limited over growth
G. virens (Gv)	–	over growth & lysis
Fluorescent pseudomonas (FP-VII)	40	–
Bacterial isolate		
(Bact-I)	30	–
(Bact-II)	45	–
(Bact-III)	36	–
(Bact-IV)	56	–
Actinomycete-V (Act-V)	Complete inhibition	lysis
Control (N. indica)	Full growth	Full growth

Table 2. Effect of culture filtrates of selected antagonists on germination of teliospores and secondary sporidia

Antagonists	Culture filtrate		Oily residue
	Teliospore germination (%)	Secondary sporidia germination (%)	Inhibition zone (mm)
Aspergillus niger (Ang-II)	1.25	22.98	–
Trichoderma koningii (Tk)	1.25	11.25	30.66
T. lignorum (Tl)	1.70	15.29	36.33
T. pseudokoningii (Tpk)	0.70	18.23	32.67
Gliocladium deliquescens (Gd)	3.65	20.50	19.67
G. virens (Gv)	2.70	15.23	29.67
Fluorescent pseudomonas (FP-VII)	1.95	12.10	16.00
Bacterial I (Bact-I)	1.95	22.60	–
Bacterial II (Bact-II)	1.35	12.98	28.33
Bacterial III (Bact-III)	2.10	15.75	22.33
Bacterial IV (Bact-IV)	2.65	8.03	27.67
Actinomycete-V (Act-V)	1.95	21.92	29.67
Control	23.00	35.80	–
CD at 5%	5.10	3.44	6.46

Table 3. Relative movement of different fractions of crude extracts on TLC plate

Antagonists	Bands/Rf value			
	A	B	C	D
Trichoderma koningii (Tk)	0.10	0.39	0.61	–
T. lignorum (Tl)	0.21	0.51	0.60	–
T. pseudokoningii (Tpk)	0.06	0.41	0.73	–
Gliocladium deliquescens (Gd)	0.20	0.46	–	–
G. virens (Gv)	0.24	–	–	–
Fluorescent pseudomonas (FP-VII)	0.22	0.45	0.77	–
Bacterial II (Bact-II)	0.15	0.30	0.58	–
Bacterial III (Bact-III)	0.18	0.42	0.72	0.95
Bacterial IV (Bact-IV)	0.32	0.62	–	–
Actinomycete-V (Act-V)	0.15	0.30	0.57	–

Apart from fungal isolates, 9 cultures of fluorescent pseudomonads and 8 each of bacterial sp. and actinomycetes were also isolated. Among these, 20 isolates showed the antagonistic potentiality against growth of N. indica on PDA and were further studied individually for their effect on mycelial growth and germination of teliospores and sporidia.

It was observed that Tk, T. harzianum (Thr), T. hamatum (Th), Tl, Tpk and G. virens (Gv) showed overgrowth and caused lysis of host-pathogen. An isolate of A. niger (Ang-II) produced limited inhibition zone (5 mm) only. Among bacterial isolates, FP-VII produced 40 mm inhibition zone, while Bact-I, Bact-II, Bact-III and Bact-IV produced 30, 45, 36 and 56 mm inhibition zones respectively. Actinomycete isolate Act-V showed complete inhibition and produced black pigment in the medium (Table 1).

Twelve isolates showing overgrowth and lysis of host mycelium as well as inhibition zone were tested for their
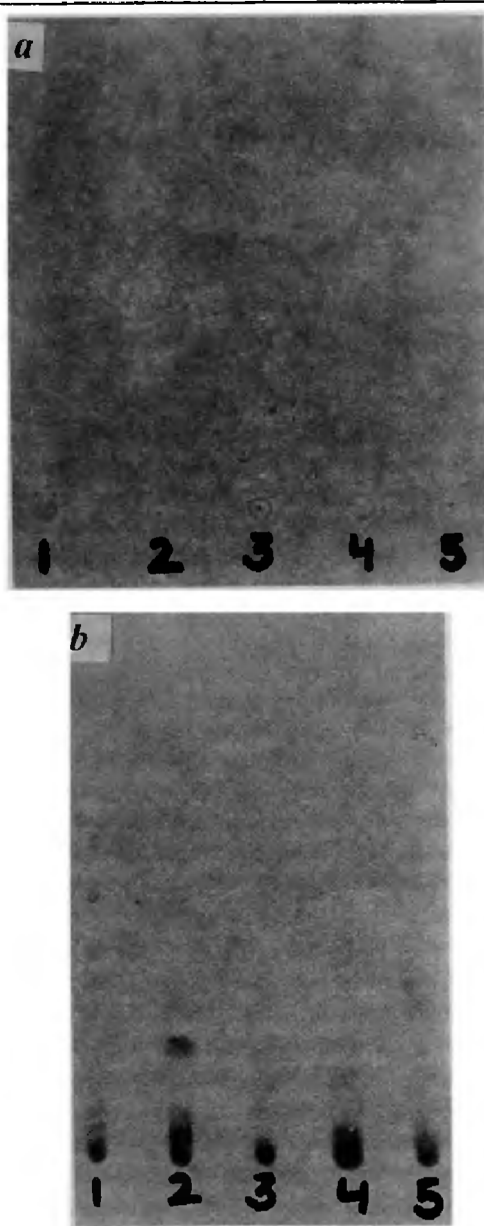
1394

Figure 1.

Table 4. Bioassay of purified antifungal compounds from selected antagonists against *N. indica*

Isolates	Inhibition zone (mm)*		
	1000 ppm	500 ppm	100 ppm
Trichoderma koningii (Tk)	24.7	13.6	0.0
T. lignorum (Tl)	29.6	19.3	12.5
T. pseudokoningii (Tpk)	22.3	10.6	0.0
Control (SDW)	0.0	0.0	0.0

*Average of three replicates.

effect on the germination of teliospore and secondary sporidia (Table 2).

The teliospore germination was significantly reduced by Tpk, Tk, Ang-II and Tl, showing 96.9, 94.5, 94.5 and 92.6% reduction over control. Among *Gliocladium* spp., *G. deliquescens* (Gd) and Gv showed 84.1 and 88.3% reduction in teliospore germination. Fluorescent pseudomonad isolate FP-VII and Bact-I, Bact-II, Bact-III and Bact-IV registered 91.5, 91.5, 94.1, 90.8 and 88.5% reduction in germination of teliospores, while actinomycete isolate (Act-V) showed 89.3% reduction.

Perusal of data from Table 2, indicated 77.6% reduction in secondary sporidia germination by Bact-IV, followed by Tk (68.6%), FP-VII (66.2%) and Bact-II (63.7%) respectively. Isolates GV, Tl, Bact-III caused reduction from 56.0–57.5%. Isolate Tpk, Gd and Act-V

proved relatively less effective showing 49.1, 42.7 and 38.7% reduction in secondary sporidia germination over control. Ang-II and Bact-I were least effective and hence these isolates were not used for bioassay studies.

The inhibition zone produced by oily residues of selected antagonists varied from 36.3 mm in the case of Tl to 16.0 mm for FP-VII (Table 2). Since the fractions of extracted culture filtrates of isolate Tl, Tpk and Tk considerably suppressed the growth of *N. indica* and produced inhibition zones of 36.3, 32.7 and 30.7 mm respectively, it was considered that they are the most effective antagonists against the pathogen. Fractionation of oily residue done on silica gel indicated the production of four bands of 0.18, 0.42, 0.72 and 0.95 Rf values. Isolates Tk, Tl, Tpk, Act-V, Fp-VII and Bact-II produced three antifungal compounds, isolates Bact-IV and Gd produced two and Gv produced only one compound (Table 3, Figure 1 *a*, *b*).

The compound produced by Tl showed 29.6, 19.3 and 12.5 mm inhibition zones at 1000, 500 and 100 ppm respectively while the compound produced by Tk and Tpk showed inhibition zones only at 1000 and 500 ppm (Table 4). It was concluded that Tl was highly effective in inhibiting the growth of *N. indica*, even at 100 ppm.

A number of *Trichoderma* and *Gliocladium* spp. have shown inhibitory effects on the growth of several plant pathogenic fungi[6]. In the present studies, Tpk, Tl and Tk showed high antagonistic activity against teliospore and sporidia germination of *N. indica*. Species of *Trichooderma* and *Gliocladium* have been reported to be antagonistic to teliospore germination and mycelial growth of *N. indica*[3]. Trichodermas have also shown antagonistic potential against other wheat pathogens such as *S. rolfsii*, *Drechslera sorokiniana*, *Fusarium* spp. and *Ustilago segetum tritici*[7–10].

In addition to fungal isolates, some bacterial and actinomycete isolates, viz. FP-VII, Bact-II, Bact-III, Bact-IV and actinomycete isolate Act-V were also found to be antagonistic to *N. indica* in inhibiting germination of teliospore and sporidia. *Bacillus* spp. and *Pseudomonas* spp. have been extensively exploited as antagonists against a number of pathogens[11–15]. Antibiosis by way of producing mycolytic enzymes/antifungal me-

tabolites has been reported to be the mechanism of biological control by many *Trichoderma*, *Gliocladium* species, bacteria and actinomycetes. Isonitrite was isolated from *T. hamatum*[16,17]; T-2 toxin from *T. lignorum*[18] and gliotoxin and gliovirin from culture filtrate of *G. virens*[19]. As seen in the present study, it is possible that purified compounds contain some antifungal elements and hence, Tl, Tpk and Tk showed inhibition of mycelial growth of *N. indica*. It is further clear from fractionation on TLC plates that isolates Tl, Gv, FP-VII, Act-V and Bact-II produced atleast three antifungal compounds.

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Chemical stimulation of germination and membrane fluidity change in secondarily dormant cucumber seeds

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Relative efficacy of various dormancy breaking chemicals is known to relate to their physical/chemical properties. Acetaldehyde required a much shorter treatment time to stimulate the germination of secondarily dormant cucumber (*Cucumis sativus* L.) seeds than ethanol and other less lipophilic compounds. T_{50} (exposure time required for 50% germination) was inversely correlated to the K_o/w (octanol–water partition coefficients) of the chemicals applied. Lipid bilayer fluidity in microsomal membranes of chemically-treated seeds was greater than in untreated control seeds imbibed in water for an equal time period. There was a significantly positive correlation between T_{50} and fluorescence anisotropy of the outer lipid monolayer in seed microsomal membranes, which indicated that membrane fluidity could be a significant event during the process of chemically-induced germination of secondarily dormant cucumber seeds.

SEED dormancy is broken by a number of organic compounds including alcohols, aldehydes and ketones[1–4]. Besides having an applied potential, they serve as molecular probes to explore the mechanisms involved in the transition from developmental arrest to growth[5]. While a number of workers[6–8] proposed a metabolic role for alcohols and related substances in stimulation of germination, others[9–11] suggested that they act at the membrane level. Interestingly, dormancy breaking activity of a large number of substances was shown to be correlated with the lipophilicity of the applied chemical[3,5]. However, all these studies focussed on the effects of chemicals on primary dormancy. Furthermore, very limited studies have been undertaken so far to correlate the effects of nonhormonal chemicals with the membrane functions[4].

Secondary seed dormancy in cucumber is known to be released by acetone, ethanol and a few other organic compounds with a concomitant change in the permeability of the cell membranes[12]. The aim of the present investigation was to determine (i) whether the dormancy-breaking activity of certain nonhormonal chemicals in cucumber is a function of their lipophilicity, and (ii) whether a change in fluidity, if any, in seed cellular membranes occurs during the chemical-induced transition from dormancy to germination in secondarily dormant cucumber seeds.

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