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Fungal metabolites from *Aspergillus niger* AN27 related to plant growth promotion

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Two metabolites have been isolated from *Aspergillus niger* AN27, a biocontrol agent, and identified as 2-carboxymethyl 3-n-hexyl maleic acid (compound 1) and 2-methylene-3-hexylbutanedioic acid (compound 2). Their biological activities related to crop growth promotion have been assayed. Both the compounds increased germination and improved crop vigour. Compound 1 was more effective for increase in germination and shoot length, whereas compound 2 had relatively greater role in increasing the root length and biomass of cauliflower seedlings.

Plant vigour is an important factor in resistance of crop to several diseases[1]. Introduced biocontrol agents, mostly bacteria only have been reported to increase plant vigour as well as yield[2-6]. Among the fungal biocontrol agents, *Trichoderma* spp. and some unidentified sporulating and nonsporulating fungal isolates have been reported to promote plant growth and suppress soil borne diseases[7-12].

Fungal metabolites related to plant growth promotion have been identified in a few cases[13-15]. Almassi *et al.*[16] have reported some secondary metabolites *viz.* 2-carboxymethyl 3-n-hexyl maleic acid anhydride, 2-methylene-3-hexylbutanedioic acid and 2-methylene-3-(6-hydroxy hexyl)-butanedioic acid which they isolated from an unspecific isolate of *A. niger*. But none of these fungi has been reported to have biocontrol ability.

Efficacy of *A. niger* strain AN27 has been tested against different soil borne fungal plant pathogens in laboratory and in field[17-19]. The principal antifungal compound has been isolated from this strain and identified as *trans* and *cis*-4(3acetoxy-6-methoxy-2-hydroxyphenyl)-2-methoxy-butanolide[20]. Besides controlling disease, the isolate also promotes growth and crop yield[18]. The present paper reports the isolation of two compounds from the biocontrol agent, *A. niger* strain AN27 and their plant growth promoting activity.

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Test crop—Cauliflower cv. Pusa snow ball K1 obtained from the Division of Vegetable Crops, of the Institute was used as the test crop for bioassay.

Isolation of secondary metabolites—Growth promoting compounds were isolated as described earlier[16]. *Aspergillus niger* AN27 was inoculated in Sabouraud broth medium (40g, dextrose; 10g, peptone in 1 litre H_2O, *p*H 5.6)@ 2ml of 2.5×10^8 cfu ml^{-1} in each of 500ml medium lots. The flasks were incubated in a shaker BOD at 150 rpm for 7days at 30^0C. The culture broth was filtered through a Whatman filter paper (No.3) and the filtrate was extracted with ethylacetate. The organic layer was separated, dried over anhydrous sodium sulphate and concentrated on a rotary vacuum evaporator. The dark brown oily residue obtained showed the presence of three metabolites on thin layer chromatography using hexane:ethylacetate:acetic acid (60:40:0.1). These metabolites were separated by column chromatography on silica gel eluting with dichloromethane to ethylacetate. The separated metabolites were characterised by nuclear magnetic resonance (NMR) and mass spectroscopy. Quantification was carried out on a thermo-separation product model spectra system P2000, high performance liquid chromatography (HPLC), equipped with variable wavelength UV-150, UV-VIS detector and a Rheodyne injector (20 µl loop). ^{1}H-NMR spectra were recorded on Varian EM 360L (60 MHz) and Bruker 400 AC (400 MHz) instruments. Deutero-chloroform ($CDCl_3$) and pyridine were used as solvents and tetramethylsilane

(TMS) as internal standard. A HRGC-MEGA2 series gas chromatograph coupled to a FISONS-TRIO 1000 ion trap mass spectrometer and connected with a Panasonic KX-P1150 multi-mode printer was used. The ionisation potential was 70 ev. Gas chromatograph was equipped with a SE -54 capillary column (15m x 0.25 mm i.d., film thickness 0.1 - 0.15 µm). GC conditions were - initial temperature 70 ^{0}C for 1 min.; and then heated up @ 10^{0}C min.$^{-1}$ to 250^{0}C for 15 min.

Bioassay—In blotter test, 2ml of compound was used at different concentrations in sterilised deionised water. Twenty cauliflower seeds were soaked for 1hr at the same dilution of the compound, placed on filter paper and incubated for 4days in a growth chamber at 27^{0}C, 78-85 % RH, and 500 µE/ m^2 for 10hr light intensity (white fluorescent tubes). Three replications were kept for each dilution.

In vial test, 7days old cauliflower seedlings grown on sterilised sand were suspended in vials (9 ml) filled with dilutions of the compounds in sterilised MS solution[21] (without sucrose and vitamins). Vials containing one seedling each, were kept in growth chamber for 15days. Ten replications were kept for each treatment.

Ethylacetate extract of culture filtrate yielded three secondary metabolites (Rf values 0.77, 0.67 and 0.57). These compounds were quantified by HPLC as 19.84 mg of compound 1, 14.56 mg of compound 2 and 0.46 mg of compound 3 per litre of culture filtrate. Compound 1 separated out as an oil and was further purified by preparative TLC. Its NMR spectra showed a triplet at δ 0.88 (CH_3), a multiplet at δ 1.3-1.6 (4 x CH_2), a triplet at δ 2.51 (CH_2 connected to a double bond) and a singlet at δ 3.51 (CH_2). Its IR spectrum showed a broad absorption band from 3600-2300 cm^{-1} for OH group and absorption band at 1720 cm^{-1} for carbonyl group. Its mass spectrum showed a molecular ion peak at m/z 240 (very weak) with base peak at m/z 222 (M^+-18). NMR spectrum of compound 2 (a white crystalline solid) showed a triplet at δ 0.83 (CH_3), a multiplet at δ 1.25 (8H, 4 x CH_2), a multiplet at δ 1.8 (CH_2), a triplet at δ 3.35 (CH), and two singlets at δ 5.80 and 6.40 for =CH_2 protons respectively and at δ 9.60, 2 proton for OH group. Its IR spectrum showed the presence of a broad band at 3600-2300 cm^{-1} for hydroxyl group and a carbonyl absorption band at 1720 cm^{-1}. Its mass spectrum showed a molecular ion peak at m/z 214 (M^+) with fragment ion peaks at m/z 197 (M^+-OH), 180 (M^+-2 x OH). On the basis of above spectral data, compounds 1 and 2 were identified as 2-carboxymethyl 3-n-hexyl maleic acid and 2-methylene -3-hexylbutanedioic acid (hexyl itaconic acid) respec-

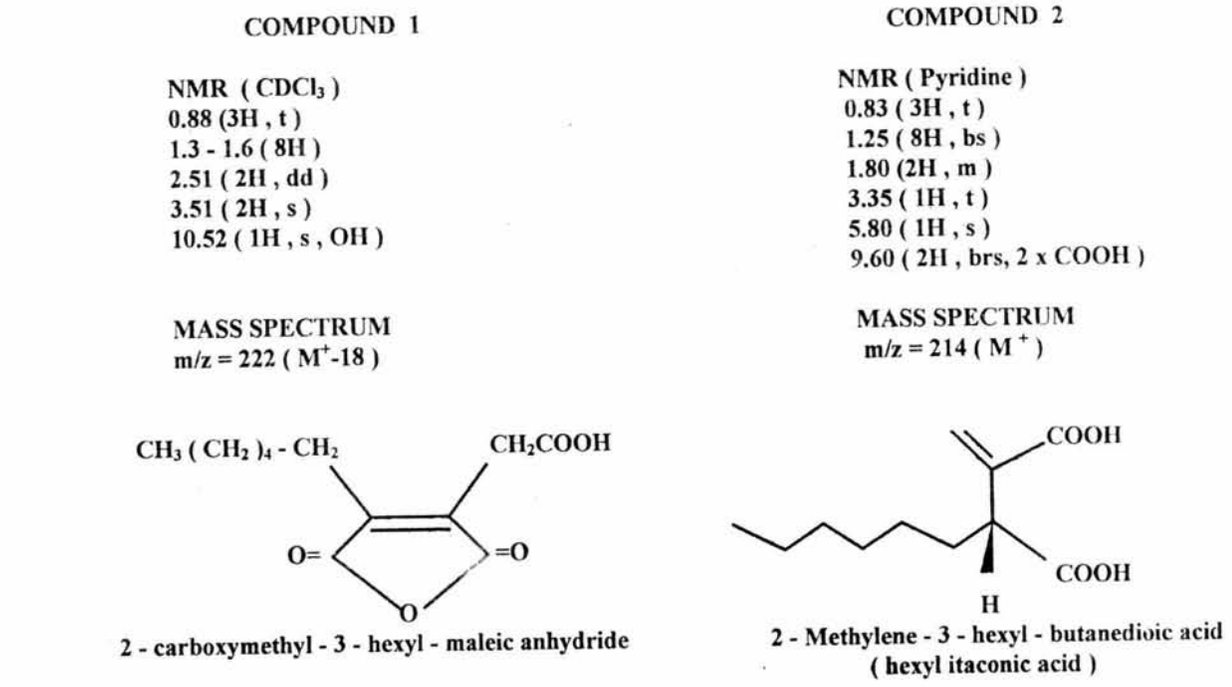

Fig. 1—Spectral analysis of two crystallised growth promoting compounds isolated from *Aspergillus niger* AN27

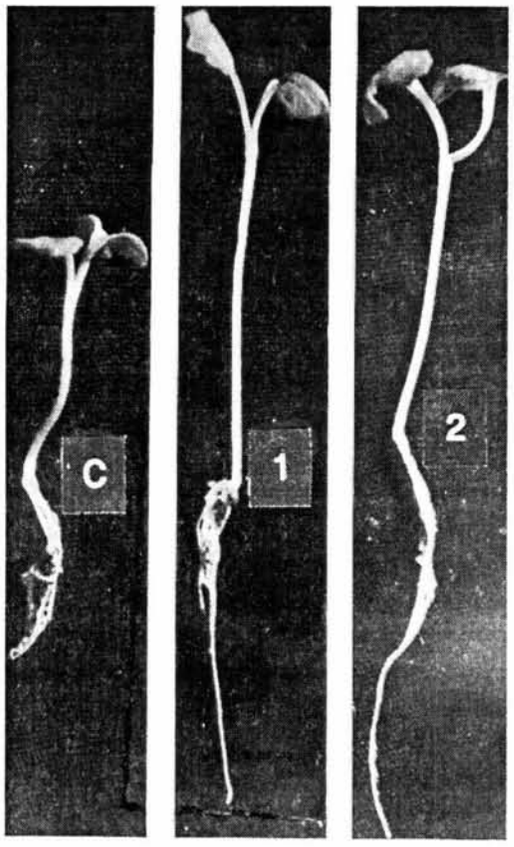

Fig. 2—Cauliflower seedlings raised from seeds: C-untreated; 1-treated with compouund 1 (10 ppm); 2-treated with compound 2 (25 ppm).

tively (Fig. 1). Compound 3 could not be identified as the isolated amount was inadequate.

On the basis of bioassays, compounds 1 and 2 were found to enhance germination and growth of cauliflower seedling (Fig. 2). The compounds 1 and 2 at 100 ppm increased germination up to 25 and 13 per cent and biomass up to 59.0 and 66.3 per cent respectively, over control. Increase in shoot length was up to 46.6 per cent at 10 ppm of compound 1 whereas up to 38 per cent at 50 ppm by compound 2. Compounds 1 and 2 increased 27.8 and 34.5 per cent root length at 10 and 25 ppm respectively. However, higher concentrations of both the compounds retarded plant growth.

On the basis of bioassay, it was concluded that compound 1 was more effective in increasing germination and shoot length whereas compound 2 was more responsible for promoting biomass and root length. Though both the compounds had been isolated from unspecific *A. niger* isolates[16,22,23], only 2-methylene-3-hexylbutanedioic acid has been quoted responsible for shoot and root length promotion in lettuce and rice[16]. Impact on seed germination and crop vigour has not been studied. Moreover metabolite production is isolate specific[16,22,23]. Fungal metabolites related to growth promotion have been identified in *A. nidulans*[14] and *Neocosmospora vasinfecta*[15]. Sassa *et al.*[13] have isolated a growth promoting compound containing radiclonic acid from an unidentified fungus. These fungi have not been reported for biocontrol activity. In this context, *A. niger* AN27 has proved itself as a mycoparasite of several important soil borne fungal plant pathogens *viz.*, *Fusarium oxysporum ciceri*, *Macrophomina phaseolina*, *Pythium aphanidermatum* and *Rhizoctonia solani in vitro*[24]. It reduces disease incidence of *Fusarium* wilt of muskmelon[17,19] and charcoal rot of potato[19,24] over 87 and 93 per cent respectively. It also increases potato yield over 18.48 per cent besides crop vigour[18,19,24]. Among fungal biocontrol agents, *Trichoderma* spp. have been studied and found to enhance plant vigour[7,8,25]. However, till now, specific compounds responsible for growth promotion have not been identified in any fungus acting simultaneously as a biocontrol agent. It can be presumed that the compounds isolated from *A. niger* AN27 may have a role in promoting yield by improving crop vigour. The strain can be used for crop protection and enhancement of production.

Toxic effects of the compounds at higher concentration inhibiting germination and retarding growth of cauliflower is a general characteristic of any growth regulator that promotes plant growth[26].

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