
C Dilip1 & B S Dilip Kumar2

1Crop Improvement Division, Central Plantation Crops Research Institute (ICAR), Kayamkulam 690 533, India. 2Soil Microbiology Division, Regional Research Laboratory (CSIR), Jorhat 785 006, India

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A hydroxamate type siderophore producing fluorescent *Pseudomonas* strain, isolated from the rhizoplane of paddy root showing plant growth promoting activity, exhibited a decreased *in vitro* antibiosis, production of siderophore and suppression of collar rot in presence of metham sodium. Use of herbicide had a detrimental effect on the plant growth promoting activity of this organism. The multiple drug resistant mutant strain derived from this rhizobacteria colonized the roots, but the herbicide application had a negative effect on their population. HPLC analysis of the siderophore showed five peaks of which the peak number three confirmed the antifungal activity.

Extensive use of herbicides for control of weeds is a great concern in the present day agriculture. Herbicides normally applied in agricultural practices are tested for their non phytotoxicity to crop plants but little attention is given to their toxicity to non target microflora including plant associated beneficial microorganisms. Some of the herbicides are known to alter the microbial *niche* in the soil and thus, several types of interaction are possible when a herbicide is introduced into the plant environment1.

Mekwatanakarn and Sivathamparam2 have provided evidence of an increased incidence of take-all of wheat by *Gaemannomyces graminis* in herbicide treated soils, suggesting that it may be due to an indirect effect of the inhibition of antagonistic microorganisms. Application of phenoxy type herbicide, mecoprop, has also been reported to have increased infection by fungus, even though it was not clear whether the effect was due to root damage or by the suppression of plant growth promoting rhizobacteria (PGPR)3.

Peanut (*Arachis hypogea* L.) is one of the most important oil yielding crops, widely grown in tropical and sub tropical and in continental parts of temperate countries. Collar rot caused by *Aspergillus niger* is a serious disease of agricultural importance to this cash crop. Earlier we had reported seed bacterization with a siderophore producing fluorescent *Pseudomonas* strain FPO4 enhanced the growth of peanut seedlings and yield, besides suppression of collar rot in field trials4. In this study we have examined the influence of metham sodium (sodium n-methyl dithiocarbamate), generally used as a soil fumigant for the control of weeds, soil fungi, nematodes and soil insects, on disease suppression, *in vitro* antibiosis, siderophore production and root colonization by this organism.

**Materials and Methods**

Organisms—The fluorescent *Pseudomonas* strain FPO4 was isolated from the rhizoplane of paddy (*Oryzae sativa* L) roots and identified as described earlier5. The pathogen *Aspergillus niger* (ATCC 9092) was procured from the national culture collection centre of IMTECH (CSIR), Chandigarh, India.

*In vitro antibiosis studies*—Petri dishes containing King's medium B6 (KB) amended with different concentrations (2.5, 5, 10 and 15 ppm) of metham sodium (procured from R&D division of Excel Industries Pvt. Ltd, India) were streaked (2 cm inside from the periphery) with an inoculum (18 hr old) of *Pseudomonas* strain FPO4 in a uniform straight line and incubated at 28±2°C. After 48 hr, an actively growing mycelial disc (4 mm) of *A. niger* was placed opposite side of the bacterial streak at a distance of 5 cm and allowed to grow at 28±2°C. Zone of inhibition (distance in mm) were measured after five
days of growth. KB plates inoculated with the bacterium and fungus without metham sodium served as control.

The length and width of the bacterial streak were recorded after 48 hr (before inoculation of fungus) and five days of growth. The growth of the fungal mycelial colony was noted after five days of growth.

Siderophore production—The organism *Pseudomonas* strain FPO4 was grown in succinate medium amended with various concentrations (0, 2.5, 5, 10 and 15 ppm) of metham sodium and the siderophore produced in the culture filtrate was determined spectrophotometrically at different intervals of growth as described earlier by Dileep Kumar and Dube.

*Extraction and bioassay of siderophore—* Was done according to Dileep Kumar and Bezbaruah. *Aureobacterium flavescens* (Arthrobacter flavescens JG 9), *Proteus vulgaris*, *Provedencia retgerri* 39 and *Morganella morgani* (obtained from Prof. Winkelmann, University of Tubingen, Germany) were used to detect the nature of the siderophore.

**HPLC analysis of siderophore—** HPLC analysis of the siderophore was done according to Berner et al. Purified siderophore extract (20 μL) was injected to a Waters HPLC system (Millipore, USA) with 486 tunable absorbance detector (UV-Vis), 510 pump, U6K universal liquid chromatograph injector, 680 automatic gradient controller with 746 data module and nucleosi-100 (C-18, 5 μm) vertex column (Saulentechnik, Berlin) and run in an isocratic mode with acetonitrile: water (60: 40) containing trifluoroacetic acid (0.1%, v/v) solvent system (flow rate 0.5mM/min) for 45 min. The peaks were monitored at 435 nm, were collected separately and tested for antifungal property against *A. niger*.

**Seed bacterization—** Seed bacterization was done as described earlier. Seeds of peanut (GGII, supplied by GROFED, Gujarat, India) were surface sterilized with sodium hypochlorite (2.4%) for 3-4 min, rinsed in sterile distilled water and dried overnight under a sterile air stream. Sterilized dry seeds (50 g) were then dipped for 30 min in bacterial suspension prepared by adding 48 hr old culture scrapped from KB medium in 50 mL of 1% carboxymethylcellulose (CMC). The seeds were then dried in sterile air for 12 hr. The dried treated seeds were sampled and colony forming units (CFU) were determined on KB medium. The bacterial suspension was adjusted to give 1.8×10⁸ CFU/seed.

Bacterized seeds were sown in plastic pots (45×45 cm) containing local field soil (silt loam, pH 7.4) infested with the fungus (10⁷ conidia/cm²) and allowed to grow under green house condition. Effect of metham sodium on disease suppression was examined by adding metham sodium (250 mL containing 2.5, 5 and 10 ppm per pot) after 2 days of sowing the seeds. Effect of different concentrations of the introduced chemical on growth was seen by measuring the length of shoots and roots, fresh and dry weight of plants and number and weight of the pods. Fifty plants randomly selected from different pots were used for recording the data.

**Suppression of collar rot disease—** Bacterized seeds grown in soil infested with *A. niger* containing different concentrations of metham sodium (as described above) were examined for the development of disease syndrome after 28 days of growth. The number of plants showing disease symptoms were scored based on the intensity of the disease severity. Average from two sets of fifty plants each taken randomly from different pots were used for recording the data.

**Root colonization—** Multiple drug resistant mutant strain of *Pseudomonas* strain FPO4, resistant to mixture of three antibiotics; ampicillin, penicillin and streptomycin (100 mg/L each), developed as previously described was used to monitor the root colonization. For this, one g of fresh root (cut in to one cm segment) taken from bacterized plants was shaken in 10 mL of sterile distilled water and different dilutions were plated on KB medium containing 100 mg/L each of a mixture of three antibiotics; ampicillin, penicillin and streptomycin. The colony forming units (CFU) were counted after 48 hr of incubation.

**Statistical analysis—** Data was subjected to Student's t test by using a computer programme developed by the S S computer centre of Bhavnagar University, India and the significance were determined at 1 and 5% levels.

**Results**

*In vitro antibiotic—* The results indicated that the bacterium in KB medium without metham sodium (ie, the control) grew well after 5 days without allowing any growth of the introduced fungal pathogen, *Aspergillus niger* (Table 1). However in presence of metham sodium, the fungus made growth and the inhibition was visible. The width of inhibition zones...
was maximum at the lowest test concentration of 2.5 ppm and it decreased with further rise in herbicide concentration. It suggested that metham sodium prevented the inhibition of the fungus by the bacterium. It was also noted that the growth of the fungus after 5 days showed a continuous increase in the diameter of the fungal colony with the rise in concentration of the herbicide. The highest growth in presence of 15 ppm of herbicide was two fold than in the medium containing 2.5 ppm. From the results, it was evident that metham sodium had no effect on the growth of A. niger, incitant of the disease.

Table 1 also shows the effect of metham sodium on the growth of the bacterium measured by length and breadth of the streak after 2 and 5 days. There was no significant difference in the size at different concentrations of metham sodium on the two days of measurements.

Influence of metham sodium on siderophore production—Data (Fig 1) suggested that metham sodium inhibited siderophore production which increased with the increase in concentration. Siderophore production was not detected up to 24 hr at 5 ppm concentration where as there was no siderophore production above these concentration. However the growth of the organism was not affected by metham sodium in succinate medium (data not given).

Bioassay and HPLC analysis—Bioassay of the siderophores with the test organisms showed that extract was unable to support the growth of all test strains except Aureobacterium flavesens JG 9, thus confirming that the extract had a hydroxamate type of siderophore. HPLC analysis of the siderophore extract showed five peaks out of which peak no.3 at a retention time of 8.59 min with 66.97 % of the total area confirmed the antifungal activity against A. niger.

Shoot height—The seedlings raised through seed bacterization showed enhanced shoot length over the non bacterized ones in soil infested with A. niger (Table 2). However introduction of metham sodium reduced growth enhancement at all the three test concentrations. The reduction in shoot length was statistically significant on all days except on 7th day at 5 and 10 ppm concentration whereas it was significant from 21st day onwards in 2.5 ppm concentration. Maximum statistically significant retardation was noted at the highest (10 ppm) concentration of metham sodium on 28th day.

Root length—Data recorded were statistically significant on all the days of observation at 10 ppm concentration (Table 2). Maximum retarding effect was noted on the 28th day, at all metham sodium concentrations.

<table>
<thead>
<tr>
<th>Growth of bacteria/fungus</th>
<th>Metham sodium concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (control)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

A - inhibition zone; B - radial growth of the fungus; C - bacterial growth after 2 days; D - bacterial growth after 5 days.

Table 1—In vitro antagonism (of growth/inhibition zone in mm) of Pseudomonas strain FP04 against A. niger on KB medium amended with metham sodium.
Fresh and dry weight—Addition of metham sodium reduced the fresh and dry weights attained through bacterization (Table 2). Reduction was proportional to the increase in metham sodium concentration. Reduction in fresh weight was statistically significant on all the days (ie, upto 28 days) of observation at 10 ppm concentration whereas the dry weight was significant only up to 21 days.

Influence of metham sodium on pod yield—Reduction in pod yield because of herbicide application was significant only at 10 ppm concentration (Table 3).

Influence of metham sodium on collar rot disease—Seed bacterization brought a distinct statistically significant decrease in the number of infected plants in absence of metham sodium (Table 4). Incorporation of the metham sodium enhanced the number of infected plants, which was proportional to the concentration of the herbicide.

The rot restricted to collar region was in the same intensity in all the treatments. However the intensity of rot in above and below regions had an effect of introduced chemical.

Influence of metham sodium on root colonization—Figure 2 shows the root colonization of bacterized peanut plants by the introduced fluorescent pseudomonad strain FP04 in A. niger infested soil containing different concentrations of metham sodium over the control soils without metham sodium. There was a fall in the CFUs at all the test concentrations from 7th to 14th day. From 21st day onwards the population steadily increased in all the cases. From 14th day onwards, decrease in root colonization was directly proportional to the concentration of metham sodium.

![Graph showing CFU counts with different concentrations of metham sodium over time.](image)

**Fig. 2.—** Influence of metham sodium on root colonization of the introduced drug resistant mutant strain of *Pseudomonas* strain of FP04 in soil infested with *A. niger*.

The data in parenthesis indicate % decrease over the control.

### Table 2—Effect of bacterization of peanut on growth in soils infested with *A. niger* and metham sodium after 28 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot height (in mm)</th>
<th>Root length (in mm)</th>
<th>Fresh weight (in mg)</th>
<th>Dry weight (in mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infested with only <em>A. niger</em></td>
<td>72.57</td>
<td>132.02</td>
<td>3180.93</td>
<td>362.57</td>
</tr>
<tr>
<td>A</td>
<td>88.94</td>
<td>177.88</td>
<td>4286.35</td>
<td>424.45</td>
</tr>
<tr>
<td>B</td>
<td>77.50*</td>
<td>144.94*</td>
<td>4147.40</td>
<td>392.75</td>
</tr>
<tr>
<td>C</td>
<td>75.13*</td>
<td>135.13</td>
<td>3854.03*</td>
<td>376.35</td>
</tr>
<tr>
<td>D</td>
<td>74.88*</td>
<td>133.63*</td>
<td>3529.90*</td>
<td>369.08</td>
</tr>
</tbody>
</table>

* Treated with *Pseudomonas* + *A. niger* + different concentrations of metham sodium (A - 0; B - 2.5; C - 5; and D - 10 ppm).

**Table 3—** Effect of seed bacterization on yield of peanut in soils infested with *A. niger* and metham sodium after 110 days.

<table>
<thead>
<tr>
<th>Yield/plant</th>
<th><em>Pseudomonas</em> strain FP04 + <em>A. niger</em> + metham sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Control)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Number of pods/plant</td>
<td>8.00</td>
</tr>
<tr>
<td>Weight of pods/plant</td>
<td>9.12</td>
</tr>
</tbody>
</table>

*Statistically significant decrease over the control (P>0.05). The values in parenthesis indicate % decrease over the control.

Concentration of metham sodium used was A - 0; B - 2.5; C - 5; and D - 10 ppm.
Table 4—Effect of seed bacterization on suppression of collar rot of peanut in soils infested with A. niger and metham sodium after 28 days of growth.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of plants infected</th>
<th>Percentage of plant parts infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Infested with only A. niger</td>
<td>29.0</td>
<td>6.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>A&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.0&lt;sup&gt;**&lt;/sup&gt;</td>
<td>6.0</td>
</tr>
<tr>
<td>B</td>
<td>20.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>6.0</td>
</tr>
<tr>
<td>C</td>
<td>24.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>6.0</td>
</tr>
<tr>
<td>D</td>
<td>28.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Only collar region; <sup>b</sup>15 mm above and below collar region; and <sup>c</sup>20 mm above and below collar region; <sup>d</sup>number of plants showed disease symptom.<sup>**</sup>Statistically significant decrease over the infested (P<0.01).

<sup>b</sup>Treated with Pseudomonas + A. niger + different concentrations of metham sodium (A - 0; B - 2.5; C - 5; and D - 10 ppm).

**Discussion**

Metham sodium did not reduced the growth of the *Pseudomonas* strain FPO4, but a reduced fungal inhibition was observed in presence of metham sodium. The suppression of siderophore production by the chemical was noted even at 5 ppm concentration. This suggests that reduction of the size of zone of inhibition in presence of the metham sodium was due to the suppression of the siderophore production. Fractions obtained from HPLC studies further confirmed the antifungal property of the siderophore.

Bioassay of the siderophore extract with test strains confirmed that the extract supported the growth of *Aerobacterium flavescens* JG 9. This confirmed that the extract had a hydroxamate type of siderophore. The absence of growth by other test strains also confirmed that the extract did not have any keto hydroxy bidentate ligands, as reported in case of *Pseudoalteromonas* Providencia and Morganella group<sup>11</sup>.

Reduction in root and shoot length of seedlings raised in fumigated pots compared to their respective control plants were proportional to the concentration of the introduced chemical. Significant loss in fresh and dry weights indicated that the chemical adversely affected the ability of the organism to suppress the pathogen. This was further confirmed with the increased number plants with collar rot in presence of metham sodium. Sensitivity of microorganisms to herbicides, including their plant growth promotion and ability to control diseases have been reported earlier<sup>11,14-16</sup>. Our findings also confirmed that metham sodium reduced the disease suppression by *Pseudomonas* strain FPO4. It is worth noting that metham sodium did not support siderophore production which contained antifungal property, and, thus, it could be concluded that its action in retarding disease suppression was by inhibiting siderophore production. Thus confirmed that siderophore was certainly a chemical determinant of disease suppression as reported earlier<sup>15-20</sup>.

Root colonization studies showed that the herbicide reduces the number of PGPR organism in herbicide treated soils as reported by Heydari et al. <sup>1</sup> in cotton rhizosphere. It has also been reported that decrease in population size may be due to change in plant root physiology especially root exudates<sup>21</sup>.

From the above results it was concluded that the application of metham sodium had a negative effect on biocontrol and plant growth promoting ability by the fluorescent *Pseudomonas* strain FPO4.

**Acknowledgement**

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**References**


