out the study. Figure 2 also shows that ambient BC level responds immediately to the resumption (even though it was only partial and confined to within the state from 21 April onwards). The strike was called off nationwide on 23 April; BC had responded immediately to the increase in the influx of trucks and reached values as high as 4500 ng m\(^{-3}\) on 24 April and continued to do so on subsequent days.

Our study has shown that the exhaust from diesel trucks contributes significantly to the ambient BC concentration at the urban centre of Hyderabad. Even though there is a sudden decrease in the BC concentration associated with the cut-off of a potential source component, the full impact is felt much later due to the finite atmospheric residence time of BC. The recovery due to resumption of truck traffic is almost instantaneous.


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**Diacetylyphloroglucinol-producing pseudomonads do not influence AM fungi in wheat rhizosphere**

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Natural agroecosystems are directly dependent on beneficial microorganisms present in bulk soil and rhizosphere for soil health and plant productivity. With the current thrust on use of microbial inoculants, especially the biological control agents, it is necessary to assess their negative influence on such beneficial forms as AM fungi since they help the plant withstand various stresses. Among other molecules, pseudomonads present in the rhizosphere secrete the antifungal, 2,4-diacetylyphloroglucinol (DAPG) and are currently in great demand as bioinoculants. Based on the cultural, biochemical, and molecular tools we show that DAPG producing pseudomonads recovered from wheat rhizosphere do not adversely affect AM colonization. Evaluation of such effects on non-target organisms will help early acceptance of microbial inoculants in future.

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**CONSIDERABLE** research efforts are underway globally to exploit the potential of fluorescent pseudomonads (FLPs) in maintenance of soil health and as crop protectants, since they represent not only a dominant bacterial group in the rhizosphere ecosystem but are also metabolically and functionally most versatile\(^{4}\). Earlier studies with FLPs in our group have provided an effective background to support exhaustive exploitation of their gene pool for the benefit of the farming community\(^{4,5}\). It is well established that FLPs effectively colonize wheat rhizosphere and exert positive influence on plant growth\(^{5}\). However, current emphasis relies on the selection of promising isolates from the rhizosphere by screening of a large gene pool for multiple functional traits\(^{6}\). Among these traits, antagonism towards phytopathogenic fungi has been studied extensively\(^{7}\). However, it is only recently that concern has been shown in field release of isolates that secrete antifungals, including, 2,4-diacetylyphloroglucinol (DAPG), since such isolates could also inhibit beneficial, naturally-occurring forms such as arbuscular mycorrhizal (AM) fungi\(^{8}\). Considering the likely negative influence of some FLPs on beneficial forms, here we describe characterization of DAPG producers from marginal wheat fields and their influence on indigenous fungi as a prelude to field use of effective isolates for improved soil health and plant productivity.
DAPG producers were isolated from three farmer’s fields in Ujjani, Dist Badnaw (Table 1). The fields were selected based on fertilizer input and grain yield and termed: (i) low input low yield (SLL), (ii) low input high yield (SLH), and (iii) high input high yield (SHH). These fields have a history of at least 20 years of continuous wheat–rice rotation and wheat variety UP2338 has been cultivated for several years. Dilution plating was performed on King’s B (KB) agar to recover rhizosphere and endorhizosphere/rhizoplane bacteria. A gene pool of 840 FLPs was recovered from the three fields. For preliminary screening, all 840 isolates were subjected to dual culture plate assay on KB : PDA (1 : 1 v/v) against Fusarium oxysporum, Helminthosporium sativum and Rhizoctonia solani. Fungal cultures were propagated on PDA for 3 days, 1 cm disk was removed and placed centrally on pre-poured (KB : PDA) plates. Bacterial cultures were spotted inoculated on the edge of the plate, and incubated at 28°C. Inhibition of fungal growth, if any, was scored after 4 days. A total of 224 gram-negative antagonistic bacterial isolates were subjected to colony hybridization to detect the presence of phiD gene.

For colony hybridization, bacterial colonies were transferred to nylon membrane lot no. IB7310 (Amersham) by standard protocol. The membranes were baked for 1 h at 42°C in 2 × SSPE (0.1% SDS, 100 µg/ml promase; 2 × SSPE is 20 mM NaH₂PO₄·2H₂O, 36 M NaCl, 2 mM EDTA). To remove bacterial cell debris from colony blot, the membranes were placed in SSPE solution without promase at 65°C with agitation. The hybridization buffer contained 5 × SSC (1 × SSC + 0.5 M NaCl, 15 mM sodium citrate), 0.1% sodium lauryl sarcosine, 0.02% SDS, and 2% blocking reagent (Boehringer Mannheim). Pre-hybridization was performed for 1 h at 52°C and hybridization was performed overnight at 52°C. The membranes were washed twice in 5 × SSC–0.1% SDS for 15 min at 52°C (hybridization temperature) and twice in 2 × SSC–0.1% SDS for 5 min at room temperature. The probe used was 17 mer PhiD1 (5’-GAGGAGCTGCAAGACCCACACAG-3’) and PhiD2 (5’-GAGGAGCTGCAAGACCCACACACAG-3’)². The probe was labelled by non-radioactive digoxigenin system using the protocol recommended by the manufacturer (Boehringer Mannheim). The hybridized probe was detected with antidigoxigenin alkaline phosphatase Fab fragment and was visualized colorimetrically using the substrate nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyolphosphate, as per suppliers instruction. DAPG-producing isolates PILH1, CHAO, PGNR1, PGNR5 and TMA3 were used as positive reference strains, and Escherichia coli and Pseudomonas aeruginosa as negative reference to check specificity of the probe.

Hybridization-positive isolates were subjected to PCR amplification of phiD gene. DNA was extracted and the first PCR was performed using the primers Phi 2a (5’-GAGGAGCTGCAAGACCCACACAG-3’ 704–723) and Phi 2b1. This was followed by second nested PCR on the 1st amplification product using primers Phi 296 (chf)/chao group 5’-CTGTCGCTACGCCGGA-3’ (202–302) Phi 296 (f) 5’CTGTCGCTATCACCACMC-3’ (286–302) in 1 : 1 mixture as forward primer and Phi 486r 5’TTRATGGA-GTTCATSAC-3’ (496–512) as reverse primer. Characterization of the isolates was done by amplifying the ITS region through Pseudomonas-specific ITS primers.

In vitro production of DAPG was tested by solvent extraction of the molecule from the culture broth and analytical HPLC. Isolates were grown in KB broth for 72 h and antibiotic extraction was carried out as described by Rodriguez and Pfender. The ethyl acetate extracts were dried in vacuum and dissolved in methanol. Aliquots were filtered (0.2 µm sartorius filter) and analysed on an HPLC system (Waters Corp Milford, MA) fitted with a PDA detector (Water’s model 501) and nucleosil C₁₈ ET-250/8/4 (Machery Nagel) column. Samples were run in an isocratic mobile phase (45% H₂O, 30% acetonitrile and 25% methanol) at a flow rate of 1.0 ml min⁻¹. The chromatogram was read at 272 nm for DAPG and spectra were compared with those of the antibiotic standard. DAPG positive isolates were functionally characterized for in vitro production of IAA and siderophore. Active culture (12-h-old) was inoculated in 10 ml minimal salt medium in 50 ml Erlenmeyer flask amended with 100 µg ml⁻¹ tryptophan. Flasks were incubated at 28°C under shaking (100 rpm) for 48 h. Culture broth was centrifuged at 7500 rpm for 10 min. To 1 ml culture supernatant, 2 ml Salkovsky reagent was added and contents incubated at 30°C for 25 min. Absorbance was read at 530 nm and levels quantitated from standard curve of IAA. For quantitation of siderophore, actively grown culture was inoculated in standard sucinate medium; inoculum size was maintained at 0.1%. Culture broth was centrifuged after 48 h growth at 28°C at 8000 rpm and absorbance of the fluorescent pigment was read at 400 nm. The amount of siderophore was calculated according to Meyer and Abdallah. Production of hydrolytic enzyme was qualitatively assayed in minimal medium containing skim milk, starch, pectin and carboxymethyl cellulose for protease, amylase, pectinase and cellulase respectively. Plates were incubated for 48 h at 28°C and formation of clear zone around bacterial colonies was clearly visible.

Table 1. Soil characteristics and distribution of antagonistic isolates in three farmers’ fields cultivating wheat variety UP2338

<table>
<thead>
<tr>
<th>Soil system</th>
<th>Available P (kg ha⁻¹)</th>
<th>Available N (kg ha⁻¹)</th>
<th>Percentage organic carbon</th>
<th>Rhizosphere</th>
<th>Rhizoplane</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLL</td>
<td>35.21</td>
<td>115.7</td>
<td>0.60</td>
<td>60.76</td>
<td>51.35</td>
</tr>
<tr>
<td>SLH</td>
<td>9.73</td>
<td>118.8</td>
<td>0.64</td>
<td>58.25</td>
<td>59.93</td>
</tr>
<tr>
<td>SHH</td>
<td>73.84</td>
<td>118.8</td>
<td>0.87</td>
<td>56.28</td>
<td>57.37</td>
</tr>
</tbody>
</table>

*Based on dual agar plate assay of 840 isolates against Fusarium oxysporum, Rhizoctonia solani and Helminthosporium sativum.
read as positive. Interaction of DAPG-positive isolates with native AM fungi was observed in a soil microcosm. Soil microcosm was prepared in plastic cups (volume, 250 g soil cup−1). The experiment was laid in unsterile soil of (a) SH1, and (b) a mixed soil (SLL + SLH, 1:1, w/w). Seeds of wheat variety UP2338 were bacterized at a population size of 10⁵ cells per seed. AM inoculum (1 g seed⁻¹) was placed in a hole and covered with a layer of soil over which bacterized seeds were sown. Each treatment was replicated thrice and arranged in completely randomized design in a net-house. Pots were watered when required. The experiment was terminated after 30 days. The bacterial population structure was assessed by dilution plating and mycorrhizal colonization by staining the roots in trypan blue. The extent of colonization was determined based on frequency distribution of fungal hyphal length in randomly selected root segments (1 cm in length). The data were analysed by one-way ANOVA.

Though soil samples were different in terms of available P and N and in organic C content, no significant variation was observed in percentage distribution of antagonistic isolates (Table 1).

Colony hybridization tests followed by two-step PCR confirmed the presence of PhID gene in six isolates (Figure 1). A 210 bp product was obtained which was also amplified in PhID-positive reference isolate, CHAO. The positive six isolates were LHRE117, LHRE30, LHRE62, LLRE63, LHR514 and HHRE81. Quantitative analysis of DAPG in liquid medium revealed that antibiotic levels ranged from 32 to 60 µg ml⁻¹. Maximum DAPG was produced in isolate LLRE63 and minimum in isolate LHRE117. Siderophore levels ranged between 20 and 30 µg ml⁻¹; isolate LHRE30 was negative for siderophore. IAA levels varied between 12 and 34 µg ml⁻¹. No specific relationship was apparent in the production between the three metabolites (Table 2). In vitro production of such secondary metabolites is not normally reflected in their efficiency to secrete these molecules in the naturally competitive environment of the rhizosphere. Thus, levels of DAPG reaching 0.33 ng have been reported in soil microcosm. In situ performance of a bacterial isolate is not only dependent on the expression of the desirable function(s), but also on its genetic background and the prevailing environmental conditions.

All six PhID⁺ isolates were able to secrete amylase and cellulase based on the relative zone size; LHRE30 produced greater amount of cellulase. Three isolates produced pectinase, viz. LLRE63, LHR514 and HHRE81, whereas five were positive for protease (Table 3).

Amplification with Pseudomonas-specific ITS primer gave a band of 1.3 kb in all isolates except LHRE30 (Figure 2), which confirmed the identity of these isolates as Pseudomonas.

The interaction of AM fungi with bacterial population in soil microcosm resulted in a positive response. At the start of the experiment, FLPs population was 10⁸ CFU g⁻¹ in both the experimental soils. After 30 days of wheat growth, FLPs population on KB reached a level of 10³−10⁸ CFU g⁻¹ soil. The total number of culturable bacteria was unaffected by inoculation with AM fungi both in rhizosphere and rhizoplane/endorhizosphere fraction. Since bacterial community analysis was not carried out, one cannot predict the change at this level. However, using fatty acid

![Figure 1](image-url). 210 bp gene product amplified after two-step PCR. Lane 1, CHAO; lane 2, LHRE30; lane 3, LHRE62; lane 4, LHR514; lane 5, HHRE81; lane 6, LHRE117; lane 7 LHR514; lane 8, Marker.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>DAPG (µg ml⁻¹)</th>
<th>Siderophore (mg ml⁻¹)</th>
<th>IAA (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHRE117</td>
<td>32.0 ± 2.0</td>
<td>20.17 ± 2.0</td>
<td>32.0 ± 3.0</td>
</tr>
<tr>
<td>LHRE30</td>
<td>44.0 ± 3.0</td>
<td>n.d.</td>
<td>31.0 ± 2.0</td>
</tr>
<tr>
<td>LHRE62</td>
<td>56 ± 2.0</td>
<td>26.28 ± 2.0</td>
<td>32.0 ± 3.0</td>
</tr>
<tr>
<td>LLRE63</td>
<td>60.0 ± 2.0</td>
<td>21.26 ± 2.0</td>
<td>34.5 ± 2.0</td>
</tr>
<tr>
<td>LHR514</td>
<td>35.0 ± 2.0</td>
<td>22.58 ± 2.0</td>
<td>25.0 ± 2.0</td>
</tr>
<tr>
<td>HHRE81</td>
<td>58.0 ± 2.0</td>
<td>38.88 ± 2.0</td>
<td>12.0 ± 1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Amylase</th>
<th>Protease</th>
<th>Pectinase</th>
<th>Cellulase</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHRE117</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LHRE30</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>LHRE62</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LLRE63</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LHR514</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HHRE81</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, Relative size of the clearing zone in plate-based assay.

Table 2. Production of DAPG, siderophore and IAA by antagonistic isolates in standard succinate medium

Table 3. Secretion of hydrolytic enzymes by PhID⁺ isolates in agar plate assay
methyl ester analysis (FAME), Gesse-Mansfeld et al. showed considerable shift in the community with the inoculation of AM fungi in Cucumis sativus. Andrade et al., have also reported qualitative change in bacterial communities as affected by different AM fungi in both the rhizosphere and hyphosphere of Medicago sativa. Mycorrhizal colonization differed with each isolate (Figure 3). An increase in the level of mycorrhization was observed from 30 to 55%. Isolates with all three functional properties and hydrolytic enzymes produced prominent vesicles in roots in the mixed soil (SLL + SLH) system. A slightly higher level of root colonization was observed in this soil. However, in an earlier study by Singh et al., it was found that levels of P had much lower effect on mycorrhization compared to root exudates which exerted the maximum influence.

Considering the known toxicity of DAPG (Table 4), the results of this study show that the negative influence of DAPG-producing FLPs on mycorrhization could be dependent on antibiotic production levels. However, available data on in vitro synthesis do not provide a clear idea of expression of antifungal metabolite in the rhizosphere. It is to be noted that mycorrhizal colonization in the presence of FLPs was not related with the level of DAPG produced by these isolates under in vitro conditions. Therefore, colonization by AM fungi may or may not be directly influenced by such bacteria. Isolate HHRE81 with maximum enzyme activity and resultant higher AM colonization could be acting as mycorrhization helper bacteria. Studies are underway to understand the influence of these isolates on AM fungi in the rhizospheric community at farmers’ field. It is, however, clear that none of the DAPG producers in this study exerted negative influence on mycorrhization colonization, which is in contrast to our earlier observation on antifungal-producing strain GRP3A which inhibits mycorrhization.

Marschner had reported negative influence of pseudomonads on AM fungi, whereas Barea et al. reported neutral influence of globally used strain P. fluorescens CHAO on AM fungi, along with those of other commonly used bioinoculants, based on Trichoderma viride and Gliocladium virens. These workers had however cautioned that introduction of a new inoculant in the soil should be preceded by detailed analysis of the biological equilibrium. Marschner et al. have recently shown that the converse is also true; AM infection altered community composition in maize rhizosphere, although synergistic interaction between AM and FLPs has also been reported. In view of the above it is essential to assess the impact of a new bioinoculant on non-target organisms and on specific microbial species, including indigenous beneficial forms so that natural biological balance can be maintained in the rhizosphere environment. In this context, several studies have demonstrated that both wild type and genetically modified Phl overproducing pseudomonads do not interfere with symbiosis, but a reliance on culture-dependent approach for impact assessment has also been questioned. There are fundamental challenges which include use of consortia for increased PGPR properties and

![Figure 2. 1300 bp ITS amplification with Pseudomonas specific primers. Lane 1, LHRE117; lane 2, LHRE62; lane 3, LLRS63; lane 4, HHRE81; lane 5, LHRS14; lane 6, LHRE30; lane 7, CHAO; lane 8, Marker.]

![Figure 3. Per cent colonization of AM fungi in the presence of Phl D' isolates in two different soils.]

<table>
<thead>
<tr>
<th>Test organism/plant</th>
<th>IC50 (μM)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>100-200</td>
<td>&gt;3200</td>
</tr>
<tr>
<td>Pythium ultimum</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>G. graminis var tritici</td>
<td>50-100</td>
<td>200</td>
</tr>
<tr>
<td>Thielaviopsis basicola</td>
<td>100-200</td>
<td>400</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>100-200</td>
<td>400</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>50</td>
<td>400</td>
</tr>
<tr>
<td>Nematode</td>
<td>200</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>


ACKNOWLEDGEMENTS. This work has been carried out under the Indo-Swiss Cooperation in Biotechnology project SA7 supported by the Department of Biotechnology and Swiss-Development Cooperation. We thank Dr Alok Adholeya and Ms Pragati Tewari, TERI, New Delhi for supply of indigenous AM consortium and laboratory support for mycorrhizal component.

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Ecobiology of the tropical pierid butterfly Catopsilia pyrane

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Successful and effective conservation management of butterflies depends on sound knowledge of their life history and host plant requirements in the wild. As part of an effort in this context of South Indian butterflies, the life history and the length of each life stage (egg, larva and pupa) of the pierid butterfly Catopsilia pyrane (Linn.), which is a strong migrant, were studied. The development period from egg to adult emergence spanned over 22–29 days, thus opening up a possibility of at least 11–12 breeding genera-

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