Cow dung extract: a medium for the growth of pseudomonads enhancing their efficiency as biofertilizer and biocontrol agent in rice

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Abstract Some pseudomonads are being utilized as biofertilizers and biopesticides because of their role in plant growth promotion and plant protection against root parasites, respectively. Two strains of *Pseudomonas*, *P. jessenii* LHRE62 and *P. synxantha* HHRE81, recovered from wheat rhizosphere, have shown their potential in field bioinoculation tests under rice-wheat and pulse-wheat rotation systems. Normally, pseudomonads are cultivated on synthetic media-like King's B and used for inoculation on seeds/soil drench with talcum or charcoal as carrier material. Cow dung is being used for different purposes from the ancient time and has a significant role in crop growth because of the content in humic compounds and fertilizing bioelements available in it. Here, cow dung extract was tested as a growth medium for strains LHRE62 and HHRE81, in comparison with growth in King's B medium. The log phase was delayed by 2 h as compared to growth in King's B medium. The bacterial growth yield, lower in plain cow dung extract as compared to King's B medium, was improved upon addition of different carbon substrates. Growth of rice var. Pant Dhan 4 in pot cultures was increased using liquid formulation of cow dung

extract and bacteria as foliar spray, compared to their respective controls. Biocontrol efficacy of the bioagents was assessed by challenging rice crop with *Rhizoctonia solani*, a sheath blight pathogen. The growth promotion and biocontrol efficiencies were more pronounced in the case of mixed inocula of strains LHRE62 and HHRE81.

Keywords Cow dung extract · *Pseudomonas* · PGPR · Bioinoculation · Plant protection

Fluorescent pseudomonads share the ability to produce plant growth promoting agents and activities, such as indole acetic acid (IAA), aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores, antibiotics and phosphorus solubilization [1]. These microorganisms are termed rhizobacteria, since their densities and activities are stimulated in the rhizosphere. Because of these qualities they alter the rhizospheric community and occupy an important niche in the root zone, exerting an important effect on plant growth promotion. They are therefore often termed “plant growth promoting rhizobacteria” (PGPR) [2].

Since the advent of green revolution, intensive use of chemical fertilizers has degraded the soil structure, composition and activities. In order to regain soil fertility by improving its texture and activities, the use of organic residues is being advocated, whereas parallel use of microbial inoculants is encouraged to achieve the required growth and reducing diseases in plants [3]. Usually, organisms sharing a number of growth promoting activities, in particular pseudomonads, were used as microbial inoculants. King's B medium was often used

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as a selective medium for the enrichment and isolation of pseudomonads. These organisms were then mass multiplied and transferred to carriers, like talc, charcoal, calcium carbonate, rock phosphate or gypsum. They were then ready to be used either by seed inoculation or soil drench after preparing a cell suspension in water. When compared to solid ones, liquid carriers proved to be better as far as shelf life and sustainability of microorganisms are concerned [4]. The present study was undertaken to find out a substrate sharing a number of advantages for its use in bioinoculation, like cheapness and availability to farmers, efficiency as a liquid carrier, as well as qualities to support growth and survival of microorganisms.

Cow is the backbone of Indian culture and rural economy. A combination of cow urine, milk, dung, ghee (fat portion) and yoghurt (curd), collectively known as Panchgavya, was used since ancient times. Every product has distinct qualities and uses in health, agriculture and other fields. Organic farming shows one of the beneficial implications of a constituent of Panchgavya [5]. Essential nutrients and pest repellents properties are obtained from cow dung, whereas urine restores micronutrients and fertility of the soil and provides food free from health hazards of chemical fertilizers and pesticides [6]. This study focuses on the use of a cow dung based extract as a growth medium and a liquid carrier. The cow dung extract was first tested for its ability to support growth of both bioinoculant *Pseudomonas* strains LHRE62 and HHRE81. Then, the effect of the bioagents inoculated in the extract was investigated for plant growth enhancement and sheath blight disease suppression in rice (*Oryza sativa* L.).

Microorganisms

Pseudomonas strains LHRE62 and HHRE81 were recovered from wheat rhizosphere of rice-wheat cropping system, under ISCB project [7–9]. They were tentatively identified to respectively *P. jessenii* and *P. synxantha* (>99% SSU-rDNA homology) [8]. These strains have been characterized for their functional properties and tested under glasshouse and field conditions for their beneficial effect on plant growth promotion.

Extract of cow dung

Composted cow dung was mixed properly with distilled water in 1:4 ratios to prepare slurry. This slurry was kept

on shaker for 12 h at 120 rpm at 28°C and filtered through double layer of muslin cloth.

Filtrate obtained through muslin cloth was processed by three ways:

- Autoclaved for 20 min at 121°C and 15 lbs
- Filter sterilized through 0.22 µm membrane filters
- Filter sterilized through 0.22 µm membrane filters followed by autoclaving for 20 min at 121°C and 15 lbs.

Growth tests

Growth of bioagents LHRE62 and HHRE81 was assessed separately on cow dung filtrate processed as above by adding 10% inoculum (0.3 OD); it was compared with growth on King's B broth inoculated in the same way. The growth curve was prepared by incubating the cultures at 28°C and 120 rpm. Log cfu/ml of culture was recorded at 1 h interval for 24 h.

Amendment of extract with carbon substrate

In order to improve growth, cow dung extract was further amended with 1% of either glycerol (G), or sodium succinate (S) or sodium citrate (C) as carbon substrates and filtered through 0.22 µm membrane filters. Seven combinations were tested: Filtrate (F), F(G), F(S), F(C), F(G+S), F(G+C) and F(S+C).

All these media were inoculated with bioagents in the same way as above to examine the efficacy of carbon substrates on their growth. Incubation conditions were 28°C temperature and shaking at 120 rpm. Data was recorded at 15 h.

Biocontrol efficacy under glass house conditions

The study was conducted on rice (*Oryza sativa* L.) variety Pant Dhan 4. Rice seeds were sown in steam sterilized clay loam soil (pH 6.50, organic carbon 1.59%, total nitrogen 0.54%, Olsen's P 10 ppm, Zn 2.43 ppm and Fe 24.53 ppm) for raising the nursery, which was transplanted after 25 days in pots of 2 kg capacity having the same soil as it was in nursery. The plants were treated with: (I) LHRE62; (II) HHRE81; (III) LHRE62 + HHRE81; and (IV) uninoculated control, both either by root dip or foliar spray. The bioagents were grown in King's B broth or cow dung extract [F(G+S)] till the log phase was achieved. Log cfu/

ml of bacterial cultures was kept constant for either broth or extract which was 10% ml. The control plants received either uninoculated broth or filtered composted cow dung extract (F). Foliar spray with the same treatment mentioned above was carried out 24 h before challenging the crop with pathogen. The sheath of plant was inoculated with one fully colonized grain of sorghum with *Rhizoctonia solani*, a sheath blight causing pathogen, after 15 days of transplantation and lesion length was measured after 5 days of challenging the plants with pathogen. The *R. solani* was multiplied on sorghum grains. The presoaked grains were taken in conical flasks (50 g/250 ml flask) and sterilized at 121°C for 20 min in an autoclave. Two disks of 1 cm diameter from a fully-grown plate of pathogen was inoculated onto the sterilized sorghum grains and flasks were kept at 27°C ± 2°C for 7 days.

Agronomic parameters

Plants were harvested after 25 days of transplantation to assess the plant vigor, i.e. shoot length and shoot fresh weight.

Statistical analysis

Agronomic parameters and disease incidence were analyzed by one way ANOVA.

King's B medium had a neutral pH, whereas the pH of cow dung extract was neutral to slightly alkaline (pH 6.9–7.9). However, this was not inhibitory for the strains studied here. When these organisms were grown in King's B media, the exponential phase started from 10 h and ended at 16 h, while using cow dung extract without carbon substrate, the exponential phase was delayed by 2 h. However, the major drawback noticed with cow dung extract was the lower growth rate compared with growth on King's B medium.

Among the three sterilization processes of cow dung extract, filter sterilization through 0.22 µm membrane filters proved to promote the highest bacterial growth rate, though it was lower than in King's B medium (Figs. 1A and 1B). The lesser growth in autoclaved sets could be due to degradation of organic compounds and subsequent release of toxic substances at high temperature.

Comparing the substrates added in cow dung extract, amendment with both glycerol and sodium succinate provided the highest cell yield (measured as colony forming units, cfu/ml) even better than in King's B medium (Fig. 2). Therefore, this medium was selected for the further studies.

Composted cow dung is rich in total nitrogen (0.14%) including some hormones and favors plant growth [5]. In general, plant vigor was found to be significantly higher with any of the treatments compared to control irrespective of mode of delivery and substrate used. The higher shoot fresh weight in control plants provided with foliar spray or root

Fig. 1A Growth curve of LHRE62. The error bars on the bullets are showing LSD ($p = 0.05$) – King's B = 0.15; autoclaved = 0.17; filtered = 0.21; and filtered and autoclaved = 0.18.

Fig. 1B Growth curve of HHRE81. The error bars on the bullets are showing LSD ($p = 0.05$) – King's B = 0.17; autoclaved = 0.14; filtered = 0.18; and filtered and autoclaved = 0.19.

Fig. 2 Colony counts of pseudomonads in cow dung extract (log cfu/ml of extract). Error bars show standard deviation. KB = King's B broth; F = cow dung extract filtrate through 0.22 mm filter; G = glycerol; S = sodium succinate; C = sodium citrate. LSD ($p = 0.05$) – LHRE62 = 0.054 and HHRE81 = 0.053.

dip of cow dung compared to King's B might because of N content, other micronutrients and hormones present in cow dung. Plant vigor was enhanced significantly when both the bioagents LHRE62 and HHRE81 were used in combination, either in amended cow dung extract or King's B broth (Figs. 3 and 4). Comparing mode of delivery of bioagents using cow dung extract, it was found that the foliar spray of a combination of both bioagents significantly enhanced shoot length by 10.5% and shoot fresh weight by 34.44% over root dip. The combination of both the bioagents delivered by foliar spray resulted in enhanced shoot length (30.21%) and shoot fresh weight (101.67%) in comparison

with uninoculated cow dung extract used as control. Nautiyal and coworkers [10] have also reported enhanced growth of maize (*Zea mays* L.), ladyfinger (*Abelmoschus esculentus* L.), rice (*Oryza sativa* L.), lauki (*Luffa cylindrica* L.) and tomato (*Lycopersicon esculentum* L.) with the use of cow dung and urine.

Used separately, each bioagent reduced sheath blight disease compared with control, irrespective of the substrate used for their multiplication (Fig. 5). However, lesion length was reduced most significantly when both the bioagents were given together, which was further reduced using glycerol and sodium succinate amended cow dung extract

Fig. 3 Shoot length (cm) after 25 days of transplantation. Error bars show standard deviation. LSD ($p = 0.05$) cow dung extract = 0.23; King's B broth = 0.22.

Fig. 4 Shoot fresh weight (g) after 25 days of transplantation. Error bars show standard deviation. LSD ($p = 0.05$) cow dung extract = 0.029; King's B broth = 0.027.

Fig. 5 Lesion length (mm) measured after 5 days of challenging rice crop with *Rhizoctonia solani*. Error bars show standard deviation. LSD ($p = 0.05$) cow dung extract = 0.97; King's B broth = 0.82.

as a substrate compared with King's B broth. Lesion length reduction was achieved by both the methods used, i.e. root dipping and foliar spray which reflected the stimulation of induced systemic resistance in plants as well as direct suppression of pathogen by bacteria used. Cow dung extract itself reduced the lesion length in either root dipping or foliar spray showing disease suppressive properties. Cow urine and dung are being used since ages in the village huts as an insect repellent. Nautiyal and coworkers [10] reported controlling of plant pathogenic fungi like *Colletotrichum capsici*, *Sclerotium rolfsii*, *Alternaria alternata*, *Penicillium species*, *R.a solani*, *Phytophthora palmivora*, *Helminthosporium* using cow dung and urine.

Our results clearly show a synergetic effect of the investigated PGPR bacteria with cow dung extract. This was strengthened by the co-inoculation of both PGPR strains. Such an optimized formulation proved to be efficient in two completely different situations: for plant-growth promotion of rice, as well as for its protection against a foliar pathogen. In both cases, inoculation by foliar spray gave better results than by root dipping. Since immemorial time, cow dung has shown its efficiency as antagonist to various diseases, and as biopesticide [6].

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