Phyllosphere-associated Methylobacterium: *a potential biostimulant for ginger* (Zingiber officinale *Rosc.) cultivation*

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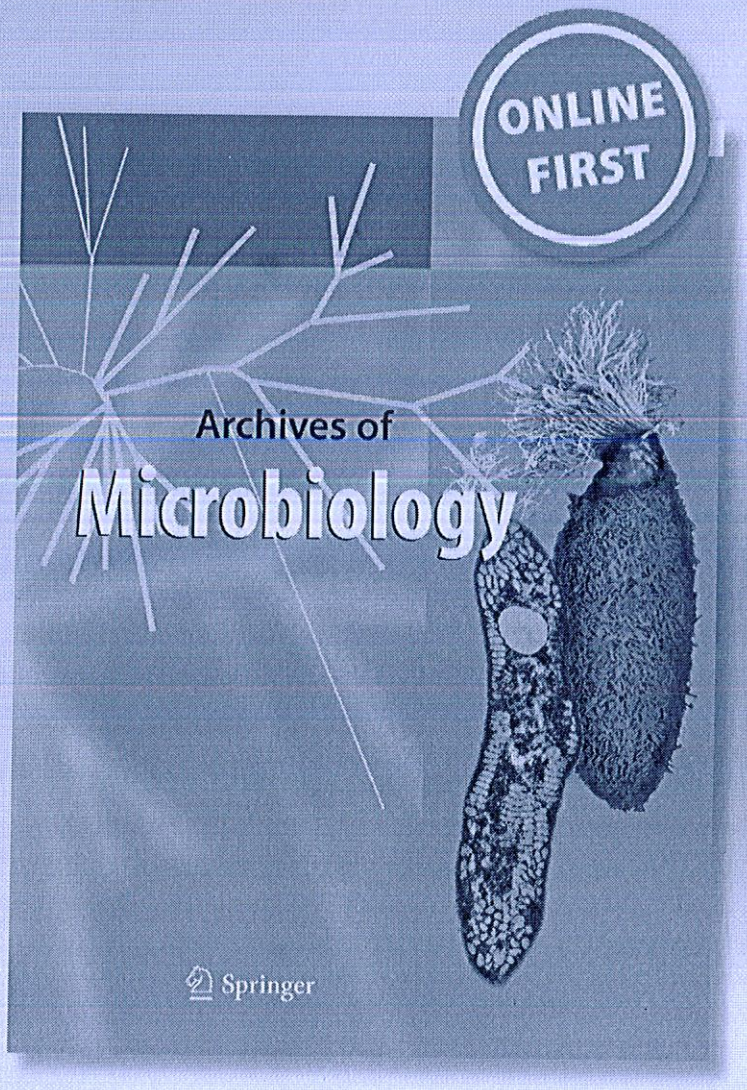

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ORIGINAL PAPER

Phyllosphere-associated *Methylobacterium*: a potential biostimulant for ginger (*Zingiber officinale* Rosc.) cultivation

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Abstract

Methanol, a by-product associated with plant metabolism, is a substrate for pink pigmented facultative methylotrophs (PPFMs) of phyllosphere. The symbiotic interaction of PPFMs has many desirable effects on plant growth and disease resistance. The present study investigated the potential of native PPFMs for mitigating biotic stress and plant growth promotion in ginger. PPFMs were isolated from ginger phyllosphere by leaf imprint technique and screened against major fungal phytopathogens of ginger viz. *Macrophomina phaseolina, Sclerotium rolfsii*, *Pythium myriotylum*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. Among the 60 PPFMs, IISRGPPFM13 was selected for its highly inhibitory activity against the target pathogens. The isolate was useful for mineral solubility, production of IAA, siderophores and hydrolytic enzymes like cellulase, pectinase, lipase, amylase and chitinase. On in planta experiments revealed that IISRGPPFM13 considerably increased plant growth parameters when the bacterium was applied as soil drenching cum foliar spraying. Methanol utilization potential of the isolate was confirmed by *mxaF* gene analysis where the sequence showing 95.51% identity towards *Methylobacterium platani* and *M. iners*. Further, 16S rRNA gene sequence showing 98.73% identity with *M. komagatae* 002-079^T (AB252201). This is the first report of its kind that a genus of *Methylobacterium* with biostimulant potential isolated from the phyllosphere of ginger.

Keywords Biofertilizer · Bioprotectant · Biostimulant · Ginger · *Methylobacterium* · Phyllosphere · PPFMs

Introduction

Ginger (*Zingiber officinalis* L.) of the family *Zingiberaceae* is one of the most widely cultivated herbaceous spice crops (Shukla et al. 2019). It is used as dietary supplements as anti-oxidant, anti-lipidemic, anti-hyperglycemic, anti-inflammatory, anti-microbial and anti-cancer activities (Silveira et al. 2018). India is the major producer of ginger contributing to around 31% of total world production. In India, Kerala stands in forefront for production of dry ginger, which has taken a major share in export (Bag 2018).

Pesticides are effective means of crop protection, but their environmental effects are diverse and they also lead to the emergence of resistance to the pathogen strains (McDowell and Woffenden 2003). Biological control is an ecofriendly, cost-effective alternative to chemical pesticides. The phyllosphere represents a heterogeneous fluctuating environment and emits methanol as the waste product of pectin metabolism during plant growth. *Methylobacterium* sp. utilize methanol as a carbon substrate for their growth and energy (Holland and Polacco 1994). The α-proteobacterial genus *Methylobacterium* is found to be the dominant group in the phyllosphere community, and reported to produces phytohormones which directly increase plant growth and productivity. Several researchers reported the growth promotional ability of *Methylobacterium* in several crops and their inoculation was found to increase the photosynthetic activity by enhancing the number of stomata and chlorophyll concentration (Ponnusamy et al. 2017). So, the present study is focused on the isolation of PPFMs from the phyllosphere of ginger and to evaluate their plant growth promotion as well as antagonistic activity against major pathogens of ginger.

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Materials and methods

Isolation and purification of PPFMs

For the isolation of ginger associated PPFMs, leaves of ginger were collected from different varieties of ginger maintained at the experimental farm at ICAR-Indian Institute of Spices Research (IISR), Peruvannamuzhi, Kozhikode and Wayanad, Kerala, India from June–November 2017. Seven different varieties of ginger viz. Athira, Karthika, Varada, Rejatha, Mahima, Aswathy and Rio-de Janeiro were selected for the isolation of PPFMs. The leaf imprinting technique was used to isolate *Methylobacterium* sp. from the plant phyllosphere (Holland et al. 2000) using ammonium mineral salt (AMS) agar amended with 0.5% methanol. The inoculated plates were incubated at 25 °C for one week. Pink colonies that appeared on the plates were isolated and re-streaked on fresh AMS media, for pure cultures. These isolates were nomenclatured as IISRGPPFMs 1–60 (giving IISR as prefix representing the Institute and G for ginger. The isolates were stored at − 40 °C and at room temperature for further studies.

Screening of antagonistic PPFMs

Major pathogens like *P. myriotylum, M. phaseolina, F. oxysporum, C. gloeosporioides* and *S. rolfsii* were obtained from the Division of Crop Protection, ICAR- IISR. It is maintained on potato dextrose agar (PDA) slants and stored at 4 °C and subcultured into fresh media as and when required. A total of 60 PPFMs were tested for their ability to inhibit the five pathogens as mentioned above by dual culture technique (Dennis and Webster 1971). PPFMs were shortlisted based on the antagonistic activity against the tested pathogens. Further growth was monitored in the AMS agar media with the range of temperature from 5 to 37 °C.

Characterization of potential PPFMs

Based on the antagonistic potential and temperature tolerance, the isolate (IISRGPPFM13) was selected and characterized as per the methods defined in Bergey's Manual of Systematic Bacteriology (Holt et al. 1994). Genomic DNA was prepared from IISRGPPFM13 and molecular identification (16S rRNA and *mxaF* analysis) were done (Jayashree et al. 2011a; McDonald and Murrell 1997). The PCR products were confirmed by 0.8% agarose gel electrophoresis. The PCR products were further sequenced at the M/s AgriGenome Labs, Kochi, Kerala, India. The obtained sequence was subjected to the EZBioCloud database search. A phylogenetic tree was constructed by a neighbor-joining DNA distance algorithm using MEGA 5.0 software (Dourado et al. 2012; Tamura et al. 2011). The nucleotide sequences were deposited in the GenBank with Accession No. MK898829.1 (16S rRNA) and MK368726.1 (*mxaF*). Solubilization of phosphate, potassium and zinc was calculated (Nautiyal 1999; Ramesh et al. 2014). Production of IAA (Khalid et al. 2004) was estimated.

In planta evaluation of potential strain

A pot culture experiment was undertaken to assess the effect of IISRGPPFM13 on growth promotion in ginger. The experiment was done under open conditions with a day–night temperature at 24–35 °C at ICAR-IISR, farm Kozhikode. Pots of 30 cm diameter were filled with potting mixture (10 kg mixture/pot) containing soil, sand and farm yard manure in 1:1:1 proportion. The treatments include drenching cum spraying with IISRGPPFM13 (T1), foliar spraying (T2), drenching alone (T3) and control without any bacterial treatment (T4) with five replications in ginger variety Varada (after surface sterilizing with sodium hypochlorite (0.5%)). Five-day-old culture (10^8 cfu/ ml) in AMS broth amended with 0.5% carboxymethylcellulose (CMC) was used as inoculum. These surface-sterilized rhizomes were dipped (bacterized) in bacterial suspension containing 0.5% CMC incubated overnight and a control was kept without seed bacterization. These bacterized rhizomes were planted @ 20–25 g/pot as prepared above and non-bacterized rhizome served as control. Culture suspension was diluted with sterilized distilled water in 1:1 ratio (Anurajan 2003) and imposed on 30-day-old plants. Bacterial suspension was applied in the morning to have uniform wetting as described by Holland and Polacco (Holland and Polacco, 1994). Foliar spray was given using a hand sprayer at the rate of 25 ml/plant. The same quantity was applied at the base of the plants as soil drenching. The treatments were repeated at 60, 90, 120, 150, and 180 days. Observations such as percentage of germination, survival, number of pseudostems, length of pseudostems, number of leaves, length of leaves, width of leaves and length of roots were recorded. Rhizomes were harvested and the fresh weight of rhizomes was recorded. Vigour Index was calculated using the formula: Vigour Index (VI) = (Root length + shoot length) *X*% germination.

Statistical analysis

The data were analyzed by an analysis of variance (ANOVA) using the statistical analysis software (SAS). Means were compared using the value of least significant difference (LSD). Significant differences between measurements for different treatments were analyzed following LSD test. $p < 0.05$ was considered statistically significant.

Results and discussion

A total of 132 native phyllosphere-associated PPFMs of ginger were isolated by leaf imprint technique using the AMS agar media amended with 0.5% of methanol as sole carbon source (Table 1). The isolates were tentatively identified as PPFMs based on the characteristic pink-pigmented colonies in AMS agar media with methanol as sole carbon source for energy (Madhaiyan et al. 2012). All the isolates were Gram negative and small rods with accumulation of PHB granules. Out of 60 PPFMs, 24 (40%) showed antagonism against *P. myriotylum,* 14 (23%) against *M. phaseolina,* 55 (92%) against *F. oxysporum*, 38 (63%) against *C. gloeosporioides* and 12 (20%) against *S. rolfsii* (Table 2). The results clearly indicated the inhibitory effect of positive isolates to *P. myriotylum* (72–79%), *M. phaseolina* (58–83%), *F. oxysporum* (62–68%), *C. gloeosporioides* (50–65%) and *S. rolfsii* (39–47%). Seven isolates showed maximum inhibition to all the pathogens tested. Based on in vitro screening, seven potential isolates (IISRGPPFM1, 2, 3, 4, 13, 59, 60) were selected based on their antibiosis efficacy against the above said pathogens. However, the isolates are having more inhibitory to *P. myriotylum* than other pathogens, showing its great potential to be a biocontrol agent against *Pythium* sp. Among the seven isolates, IISRGPPFM13 showed a wide adaptability to temperature (5–37 °C) in AMS media where room temperature being the optimum, this strain was selected for further studies. Morphologically the isolate is pink pigmented, Gram negative, aerobic, circular, raised colonies with entire margin with the inclusion of PHB granules and biofilm formation. A string was formed during the KOH test indicating that the isolate is Gram negative. The methanol tolerance level of IISRGPPFM13 was 0.5 to 2%, they are positive for catalase, oxidase, oges–Proskauer (VP) and negative for methyl red (MR) test. The isolate produced a yellow pigment in the nutrient agar, Jensen agar and tryptic soya agar (TSA) medium. It also utilized various substrates such as pectin, chitin, starch, cellulose and tributyrin. All these results are consistent with the many phenotypic characteristics of the genus *Methylobacterium* (Madhaiyan et al. 2012).

Methanol dehydrogenase (*mxaF*) gene is the main niche to assess the diversity of the genus *Methylobacterium* and also observed the *mxaF* gene association with the metabolism of PPFMs which is responsible for the plant fitness during the epiphytic colonization under stress conditions (Sy et al. 2005). Besides, the widespread occurrences of

Table 1 Details of sample and PPFMs

Location of the sample collection	Latitude / longitude	Variety	Code of the isolates (IISRGPPFM)
ICAR-IISR, Calicut, Kerala	22°93′N and 88°53′E	Athira, Mahima, Varada, Rejatha, Aswathy, Karthiga	1–4 30–60
ICAR-IISR experimental farm, Peruvannamuzhi, Kerala	11°56′N and 75°85′E	Rejatha, Varada	5–14 20–29
Wayanad, Kerala	11°68′N and 76°0′E	Rio-de Janeiro	15–19

ICAR-IISR Indian Council of Agricultural Research–Indian Institute of Spices Research; isolates were nomenclature as IISRGPPFMs 1-60 (giving IISR (Institute) and G for ginger as prefix for each isolated culture)

Table 2 Screening PPFMs for antifungal activity

Phytopathogens in ginger	Number of positive isolates	Potential isolates of IISRGPPFMs for antifungal activity							Growth inhibition (%)
		1	2	3	4	13	59	60	
P. myriotylum	24 (40%)	+++	+++	+++	+++	+++	+++	+++	72–79
M. phaseolina	14 (23%)	+	+	+++	+++	++	+++	+++	58–83
F. oxysporum	55 (92%)	+	+	+++	+++	+++	++	++	62–68
C. gloeosporioides	38 (63%)	+++	+++	++	++	+++	+++	+++	50–65
S. rolfsii	12 20%)	+++	+++	+++	+++	++	+++	+++	39–47

+ low, ++ moderate, +++ high

Percent inhibition of test pathogen was calculated using the formula: I = C-T/Cx100 where I = percent inhibition of test pathogen, C = growth of the pathogen in control and T = growth of the pathogen in dual culture

carotenoids are in PPFMs also serving as an important taxonomic marker for the identification of the isolates. The 16S rRNA (1342 bp) and *mxaF* gene (534 bp) sequences were used for studying bacterial phylogeny and taxonomy. To identify the IISRGPPFM13, representative sequences from BLAST analysis were retrieved and aligned using CLUSTAL W. Considering single carbon utilization by the isolate while expressing genetic similarity to the *mxaF* gene showing 95.51% towards *Methylobacterium platani* and *M. iners* (Fig. 1) whereas 16S rRNA sequence analysis showed 98.73% identity with *M. komagatae* 002-079^T (AB252201) (Fig. 2).

The antagonistic activity of IISRGPPFM13 against the pathogens was assessed using the dual culture plate assay (Fig. 3). The highest inhibitory effect (75%) was observed against *P. myriotylum* and the least (40%) was observed against *S. rolfsii*. The percent inhibition on other pathogens was found to be 67, 59 and 60 against *M. phaseolina*, *F. oxysporum* and *C. gloeosporioides*, respectively. IISRGPPFM13 was found to be a potent phosphate, potassium and zinc solubilizer by showing clear halo zone around the

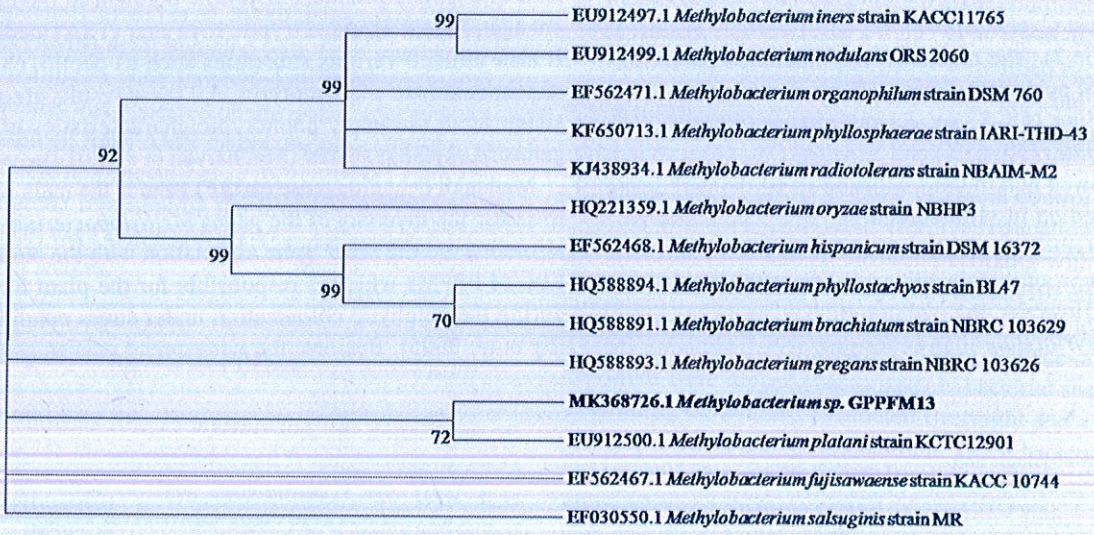

Fig. 1 Phylogenetic tree showing the evolutionary position and relationship based on *mxaF* sequences between *Methylobacterium* sp. IISRGPPFM13 and other related bacterial isolates. The tree was constructed by maximum likelihood method with 1000 bootstrap replicates

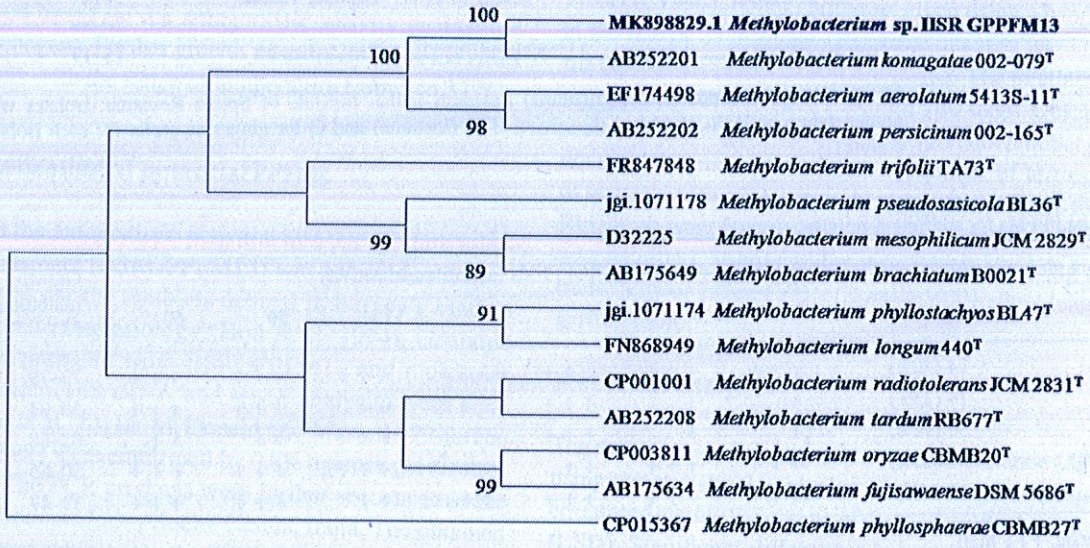

Fig. 2 Phylogenetic tree showing the evolutionary position and relationship based on 16S rRNA sequences of *Methylobacterium* sp. IISRGPPFM13 with other bacterial isolates. The tree was constructed by neighbour joining method with 1000 bootstrap replicates

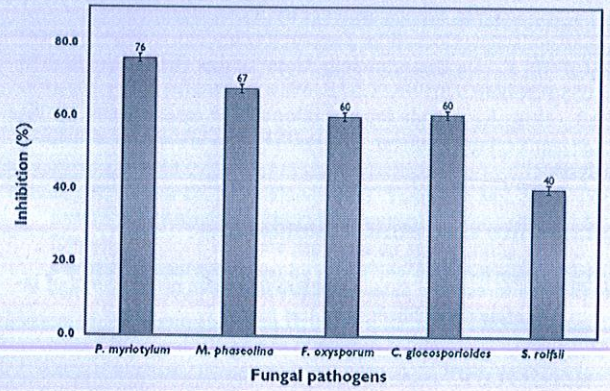

Fig. 3 Antifungal activity of *Methylobacterium* sp. IISRGPPFM13 against major pathogens of ginger. Values are significantly different according to the LSD test ($p=0.05$) in all treatments. The data are expressed as the average of three replications

Table 3 Mineral solubilization efficiency of *Methylobacterium komagatae* sp. IISRGPPFM13 strain

Minerals	Total diameter (cm)	Colony diameter (cm)	Solubility index (SI)
Potassium (K)	$6.2^{c,b}$	1.2^{b}	5.2^{b}
Phosphate (P)	7.4^{a}	1.5^{a}	4.9^{c}
Zinc (Zn)	7.0^{b}	1.3^{b}	5.4^{a}

SI = colony diameter + diameter of halo zone/colony diameter

Means with at least one letter common are not statistically significant using Fisher's least significant difference (LSD at 0.05%). The data were expressed as the average of three replications

colony. The SI of the strain was also calculated and it is 4.9, 5.2 and 5.4 for phosphate, potassium and zinc, respectively (Table 3). This strain showed clear zone of solubilization (in cm) of 5.9, 5.0, and 5.7 respectively for phosphate, potassium and zinc. Solubilization of minerals can be accomplished by a range of mechanisms, which include excretion of metabolites such as organic acids, proton extrusion, or production of chelating agents (Rodríguez and Fraga 1999). This solubilization property is important in nutrient cycling. The halo zone formation around the bacterial colonies could be due to the secondary metabolites (Paul and Sinha 2013). *Methylobacterium* sp. has the ability to dissolve inorganic phosphates and therefore may be involved in phosphate metabolism in both microorganisms and plants (Agafonova et al. 2013; Jayashree et al. 2011b). The amount of IAA produced with and without supplementation of L-tryptophan by the strain IISRGPPFM13 was determined as 7.5 and 11.1 µg/ml, respectively. IAA (µg/ml) production was reported by *Methylobacterium* sp. strains in the range of 1.1, 2.3 and 2.4. Our results indicated that IISRGPPFM13 is producing

Table 4 Various treatments with *Methylobacterium komagatae* sp. IISRGPPFM13 strain

	G (in %)	NOP	LOP (in cm)	NOL	LOL (in cm)	WOL (in cm)	LOR (in cm)	VI	RW (in gms)	Survival (%)
T1-control (no seed treatment)	83^{b}	1^{c}	61^{c}	16^{c}	22^{b}	3^{c}	11^{d}	5995^{d}	54^{d}	20^{d}
T2-drenching	100^{a}	4^{c}	82^{b}	22^{b}	24^{b}	3^{b}	19^{c}	10133^{c}	120^{c}	60^{c}
T3-spraying	100^{a}	16^{b}	94^{b}	25^{ab}	30^{a}	4^{a}	35^{b}	12867^{b}	343^{b}	100^{b}
T4-drenching with spraying	100^{a}	23^{a}	116^{a}	29^{a}	33^{a}	4^{a}	48^{a}	16367^{a}	375^{a}	100^{a}

Means with at least one letter common are not statistically significant using Fisher's least significant difference (LSD at 1%). The data were expressed as the average of five replications

G germination, *NOP* number of pseudostems, *LOT* length of pseudostems, *NOL* number of leaves, *LOL* length of leaves, *WOL* width of leaves, *LOR* length of roots, *VI* vigour index, *RW* rhizome weight, *cm* centimeter, *gms* grams

higher amounts of IAA (7.5 µg/ml) than that reported by Omer et al. (2004). It is also having the ability to produce siderophore as an indicator of biocontrol efficiency as well as production of enzymes like, cellulase, pectinase, lipase, amylase and chitinase. Many researchers reported the same that, the genus have the ability to produce the growth hormones and mycolytic enzymes, which involved in resistance mechanism and also nitrogen fixation (Jayashree et al. 2011a; Madhaiyan et al. 2006).

A genus of *Methylobacterium* is abundantly present in the all parts of the plants and promotes growth and development of the host plant by producing the variety of phytohormones (Mizuno et al. 2013). Drenching cum foliar spray showed significant increase (%) in germination (17), number of pseudostems (96), length of pseudostems (47), number of leaves (45), length of leaves (33), width of leaves (25), length of roots (77), vigour index (63), survival rate (80) and ginger yield (86) compared to the control. As a whole enhanced plant growth was observed in the three treatments along with seed bacterization (Table 4). In general, the treatment with IISRGPPFM13 led to significant increases in plant growth as measured by all the parameters. The efficacy of *Methylobacterium* strain in improving plant growth is established. In the present investigation, it is confirmed that IISRGPPFM13 inoculation to ginger plants resulted in significantly higher plant growth parameters. This may be due to the production of higher amounts of plant growth regulators. The effect of seed bacterization followed by spraying cum drenching, considerably enhanced the seedling vigour index (16,367), over the control (5995) which was significantly higher than that reported by Meena et al. (2012) where a vigour index value of 1022 only was obtained for the *Methylobacterium* sp. (NC4) isolated from sugarcane. The strain *M. komagatae* IISRGPPFM13 can be further studied for its efficacy as a bioprotectant for its application in plant disease resistance for sustainable cultivation of ginger.

Conclusions

This is the first report of a *Methylobacterium komagatae* sp. isolated from the phyllosphere of ginger as a biostimulant. It has shown inhibitory effects on major fungal pathogens of ginger especially *P. myriotylum* causing soft rot. Besides producing siderophores contributing towards biocontrol activity, production of important enzymes and also mineral solubilizing capacity make it as a better source of biostimulant, biofertilizer and as well as a biocontrol agent. This study suggests that *Methylobacterium komagatae* IISRGPPFM13 can be exploited as a promising biostimulant for sustainable ginger cultivation.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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