PLANT SIGNALING & BEHAVIOR
2019, VOL. 14, NO. 6, e1596716 (7 pages)
https://doi.org/10.1080/15592324.2019.1596716

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RESEARCH PAPER

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Piriformospora indica promotes the growth of the *in-vitro*-raised *Cymbidium aloifolium* plantlet and their acclimatization

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ABSTRACT

Cymbidium aloifolium is known for its ornamental and medicinal values. It has been listed as threatened orchid species. In this study, *in vitro* propagated *C. aloifolium* plantlets were interacted with the *Piriformospora indica*. The growth assay was performed for 45 days; the plant growth pattern such as number and length of roots and shoots were measured. Microscopic study of the root section stained by trypan blue was done to detect the peloton formation. The methanol extracts of the fungal colonized plant as well as uncolonized (control) plant were prepared and various metabolites were identified by gas chromatography mass spectroscopy. Acclimatization was done in a substrate composition of coco peat: gravel: charcoal in ratio 2:2:1. *P. indica*-colonized plantlet showed the highest growth with the formation of clamdospore in the root section. The growth regulator such as auxin, ascorbic acid, andrographolide, hexadecanoic acid, and DL-proline were identified. After three months of field transfer, plantlet colonized by *P. indica* survived and remained healthy as compared to uncolonized control plantlet.

ARTICLE HISTORY
Received 1 January 2019
Revised 11 March 2019
Accepted 12 March 2019

KEYWORDS
Piriformospora indica; *Cymbidium aloifolium*; acclimatization; symbiosis; bioactive compounds

Introduction

Cymbidium aloifolium is commonly known as aloe-leafed *Cymbidium* having both ornamental and medicinal values.[1] It is widely distributed from dry lowland tropical to subtropical region of central and eastern Nepal.[2] It has distinct characteristics that make them different from other orchid species. They have ovoid pseudobulb, leathery bright leaves and basal inflorescence with persistent yellowish flower with reddish purple chestnut radial stripes.[3] The persistent bright yellowish flower makes the *C. aloifolium* one of the highly traded flower plants.[2] Moreover, the presence of the bioactive compounds such as dihyrophenanthrene, phenanthraquione, alkaloids, phenolic compounds and coumarin adds to its medicinal property.[4,5] *C. aloifolium* is used in traditional medicine as tonic as well as in medicine to treat various human diseases and disorders.[6,7] Therefore, this orchid species can be a potential candidate in modern medicine system for the development of drugs and therapeutic agent.[8] Hence, the plants have a great potential to serve the mankind.

Orchid seed lack endosperm and are hard to germinate in nature. Only a few percent of the microscopic seeds can get a chance to germinate with the help of mycorrhizal association.[9] This may also lead to a decline in the orchid population in natural habitat apart from human activities. However, in recent years, overexploitation of the forest and illegal trade have caused a serious threat to the existence of all orchid species.[10]

Plant tissue culture technique provides the alternate way to conserve the orchid species.[11–13] However, *in -vitro*-grown plants typically orchids suffer both abiotic and biotic stresses during the hardening and field transfer process. In this regard, the *P. indica* can be used as biological tool for the growth and development, immunity during the process of hardening, and field transfer.[14] As the *C. aloifolium* have become one of the threatened and valuable species for the commercial purpose, the present investigation develops the strategy to conserve the *C. aloifolium* by exploring the potential of *P. indica* to be used as a biological tool to enhance the acclimatization process. *P. indica* is well known for its plant root colonizing activities providing nutrient and defense mechanism to overcome the biotic and abiotic stress. Previous research work shows its ability to promote plant growth of a wide range of plant species belonging to monocot and dicot. Moreover, there are reports about its ability to colonize and promote the growth of the orchid species.[15,16] Hence, the present study aims to achieve the interaction of *in vitro* cultured *C. aloifolium* with *P. indica* for the plant growth providing tolerance to overcome the abiotic and biotic stress when transfered to the natural environment.

Result

Plant growth assay

One-month-old *C. aloifolium* plantlets grown in *in vitro* condition were used for the interaction with *P. indica*. After 45 days of interaction, the significant differences in the growth pattern of the uncolonized and colonized plantlet were observed (Figure 1). The plantlet colonized by *P. indica* attained the highest growth in terms of root and shoot numbers as well as root and shoot length. (Figure 2). The histo-chemical assay of colonized root

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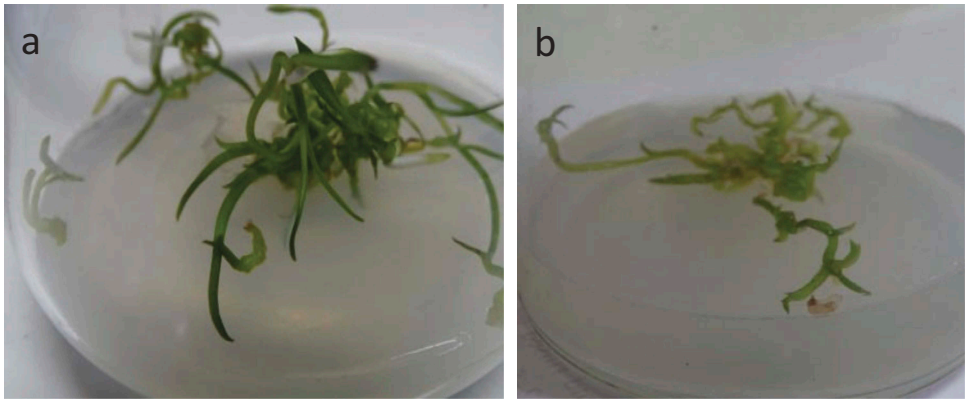

Figure 1. *In-vitro* plant growth assay of *Cymbidium aloifolium* colonized by *P. indica* (a) compared to uncolonized plantlets, control (b) for 45 days of growth assay.

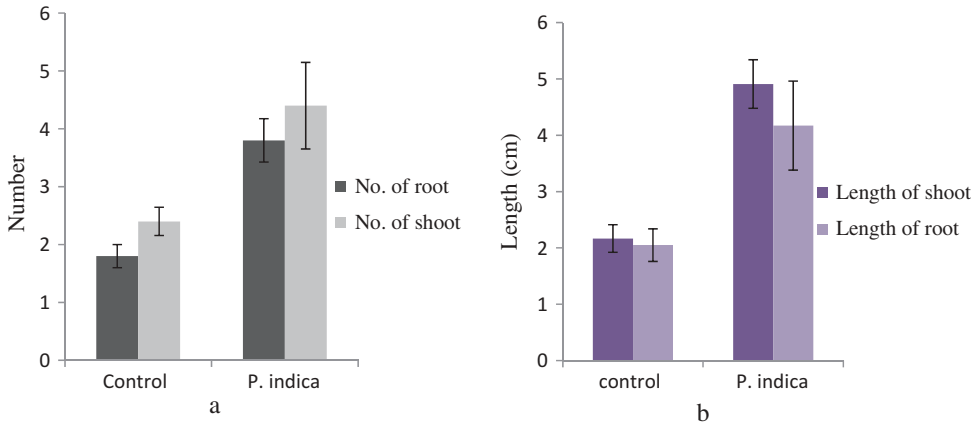

Figure 2. Morphological changes in growth pattern of the *P. indica*-colonized plantlet versus uncolonized plantlet in terms of mean of roots and shoots number (a) as well as mean of root and shoot length (b). Bar represents mean ± SE (n = 15). The data is significant at the level of $P \leq 0.05$.

section showed the presence of clamdospore of the *P. indica* in the cortex region of the root tissue. Plant growth assay confirms the formation of mycorrhizal association between the fungus and plantlet (Figure 3).

Comparative characterization of bioactive compounds of uncolonized and colonized plants

The metabolites present in the methanol extract of the uncolonized and colonized *C. aloifolium* are depicted with their reported biological function in Tables 1 and 2, respectively. The plant extracts were prepared in methanol and filtered by Whatman filter paper. The bioactive compounds present in colonized plant methanol extract as well as in uncolonized plant methanol extract were identified by GC-MS technique. The presence of bioactive compounds in the uncolonized and colonized plant extracts differed significantly. The compounds such as indole acetic acid, ascorbic acid, DL-proline, and andrographolide were detected in the colonized plant extract. These bioactive compounds were responsible to promote the plant growth and development. Ethyl iso-allocholate, mannitol, and hexadecanoic acid were detected in the extracts of uncolonized plants.

Hardening and acclimatization of uncolonized and colonized plantlet

Pre-acclimatization procedures and pot assay were carried out to measure the survival of the colonized plants compared to uncolonized plants. In this regard, different compositiond of cocopeat, gravel and charcoal were considered. The plantlets colonized by *P. indica* were able to adapt to the natural environment condition overcoming all the abotic and biotic stresses. In the present study, five individual colonized plants and five uncolonized plants (control) were transfered to the field. The substrate compositions used were cocopeat (P): gravel (G): charcoal (C) (PGC) in the ratio of 2:2:1. New roots were formed during hardening, and root and shoot lengths attained higher growth compared to control (Figure 4). The growth of the control plantlet was not significant, and leaves and roots decayed during acclimatization.

Discussion

P. indica is known to show a broad spectrum of root colonizing capacity that includes both dicot and monocot plant species.[28,29] *P. indica* provides resistance toward abiotic and biotic stress to the colonized plant species.[30,31] Previous reports clearly explain its unique ability to

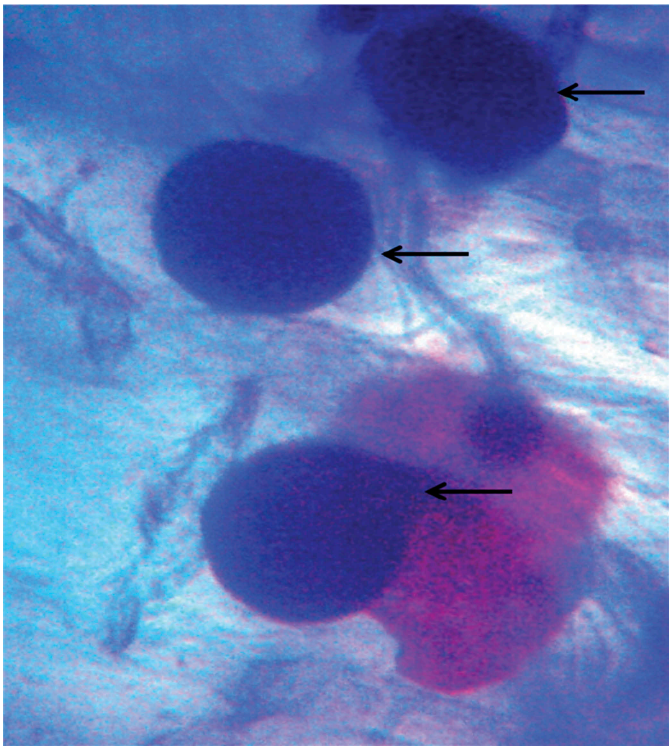

Figure 3. Microscopic view showing the formation of clamdospore in the cortical section of root tissue (arrow indicates the clamdospore formation inside the cortex region of root section).

colonize the roots of several plant species and help in the production of plant phytohormone and growth regulator such as auxin, cytokinin and jasmonic acid.[30–32] *P. indica promote* orchid seed germination as well help in growth and development. The reports on seed germination of *Dactylorhiza majalis* by *P. indica*[15] and *Oncidium* plantlet growth promotion activity are the fine examples of *P. indica* interaction with orchid species.[16] There are several examples of the *P. indica* being a root-colonizing endophytic fungus. The major goal of the present study was to investigate its ability to colonize the *in-vitro*-grown *C. aloifolium* with *P. indica*, contributing to fitness, plant growth, and development during the field trial. The formation of the clamadospore in the cortical cells of root section showed its ability to colonize the plantlet. The ability to colonize the root is beneficial for plants for nutrient uptake. This has been proven in plant growth assay (Figures 1 and 2). The plant growth assay showed its ability to promote the plant growth and development significantly as compared to the control. The present result shows similarity with the previous study of Chinese cabbage plantlet colonized by *P. indica*.[14] The overall metabolomic study of *P. indica* colonized Chinese cabbage demonstrated the number of metabolites, intermediate product, and their precursor. These metabolites were GABA, oxylipin-family compounds, polysaturated fatty acid, auxin, and its intermediate such as L-tryptophan, indoleacetamide, and indoleacetonitrile. Moreover, they were successful to show the increase in saturated fatty acids and the decreased in unsaturated fatty acids, which is an important phenomenon to combat abiotic and biotic stress.[33–35] Whereas the present study shows the ability of the *P. indica* to produce auxin on MS media supplemented with auxin precursor tryptophan. It indicates the role of P. indica to produce auxin from tryptophan supplement and helps the colonized plant to grow by providing hormones and nutrients effectively. Apart from its ability to synthesize auxin molecules, Table 2 suggests its ability to produce bioactive compound, mainly ascorbic acid, which plays an important role in photosynthesis, biochemical pathway, as well as possesses antioxidant property.[23,24] Detection of DL-proline and hexadecanoic acid in the *P. indica* colonized *C. aloifolium* shows the ability of the *P. indica* to provide resistantance against abiotic and biotic stress.[17,20] Moreover, *P. indica* also enhances the medicinal values of the colonized plant. Bioactive compound andrographolide known for its anticancer property was detected in the colonized plant. These evidences show that the *P. indica*

Table 1. Bioactive compound present in methanolic extracts of the control plantlets (uncolonized).

Peak	Retention time	Name	Mass peak	Base peak	Reported biological function
1.	10.410	Hexadecanoic acid, methyl ester	640	74.10	Antimicrobial activity[17]
2.	12.930	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	588	57.15	Antimicrobial activity[17]
3.	13.580	D-Mannitol, 1-O-(22-hydroxydocosyl)-	613	73.10	Role in abiotic and biotic stress[18]
4.	17.850	Ethyl iso-allocholate	685	55.10	Apoptotic activity[19]
5.	25.975	Beta-carotene	820	55.10	Plant biological proecess such as photosynthesis and antioxidant acitvitities

Table 2. Bioactive compound present in methanolic extracts of the *P. indica*-colonized plantlets.

Peak	Retention time	Compound name	Mass peak	Base peak	Reported biological function
1.	6.333	DL-proline, 5-0xo-,methyl ester	604	84.10	Drought-stress tolerant[20,21]
2.	7.400	Hexadecanoic acid, methyl ester	601	74.10	Antimicrobial activity[17]
3.	9.985	1H-Indole-3-acetic acid, methyl ester	644	130.15	Plant growth and development, root initiation and elongation[14,15,22]
4.	10.715	l-(+)-Ascorbic acid	637	73.10	Important factor in biochemical pathway, biological processes such as photosynthesis[23,24]
5.	19.895	Andrographolide	702	107.15	Metabolites having anticancer and antioxidant activities.[25–27]

provides fitness in *in-vitro* plant, promotes plant growth and development, as well as adds medicinal values to the *C. aliofolium*. Metabolites such as DL-proline and hexadecanoic acid present in *P. indica* colonized *C. aloifoium* confirms the ability of *P. indica* to show drought, fungal infection resistance during hardening, and acclimatization process (Figure 4). The *P. indica* colonized plantlets were able to adapt easily as compared to control attaining high growth (Figure 5). Apart from this, *C. aliofolium* has medicinal values, the detection of the ethyl iso-allocholate

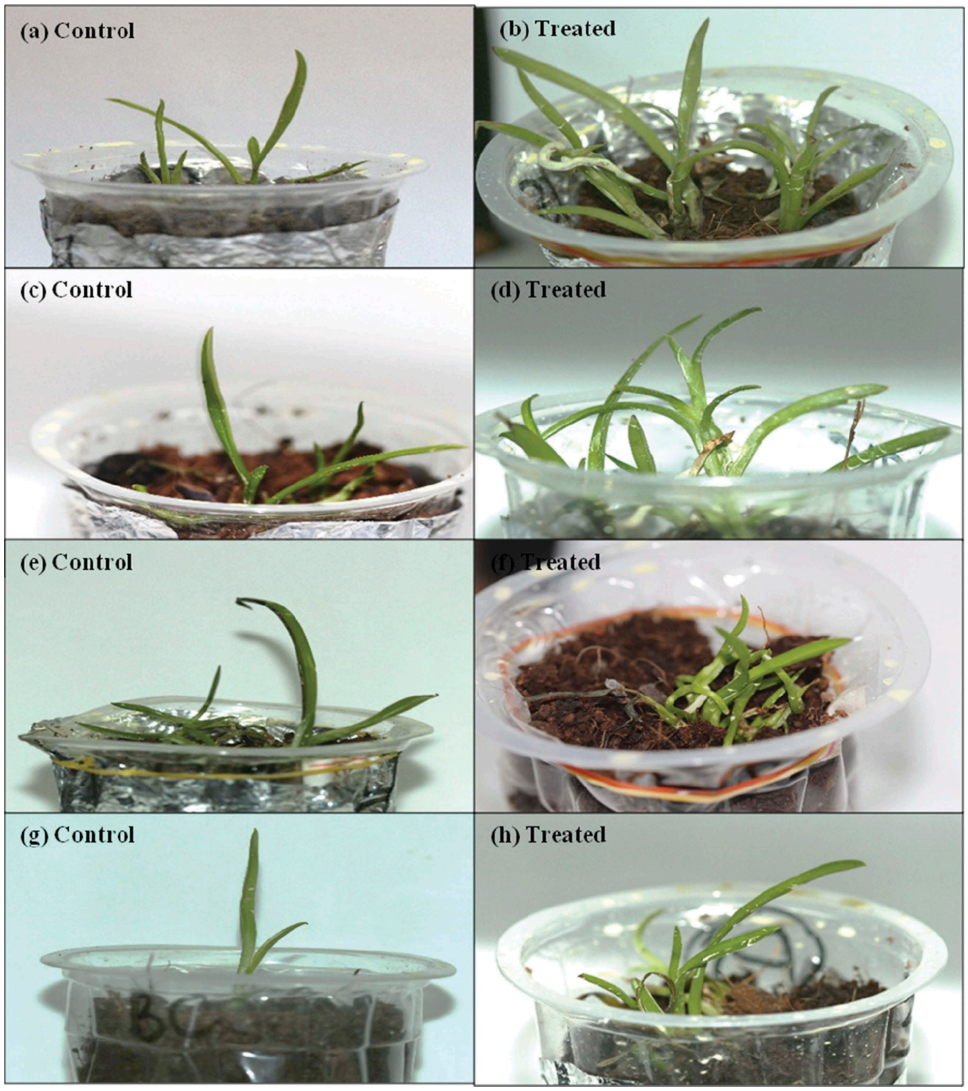

Figure 4. Acclimatization and hardening of the *P. indica*-colonized plants and uncolonized plants for 3 months using different compositions of substrate at different ratios. (a) Control. (b)Treated. (c) Control. (d) Treated. (e) Control. (f) Treated. (g) Control. (h) Treated.

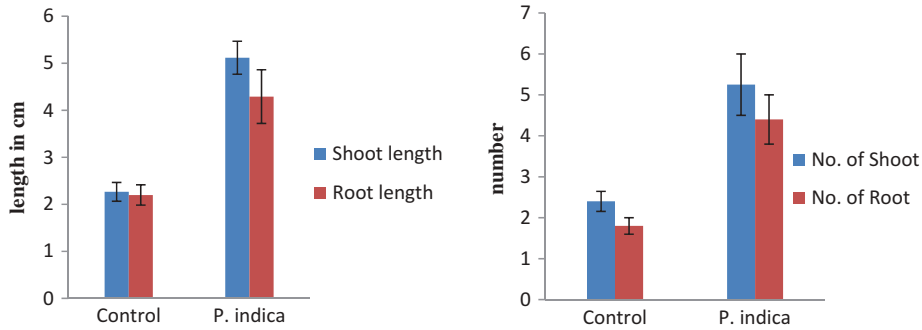

Figure 5. Morphological changes in growth pattern of *P. indica*-colonized plantlet versus uncolonized plantlet (3-month-old) after acclimatization in terms of mean of roots and shoots number (a) as well as mean of root and shoot length (b). Bar represents mean ± SE (n = 15). The data is significant at the level of $P \leq 0.05$.

compound in the extract of *in-vitro*-grown uncolonized plantlet extract. The presences of ethyl iso-allocholate in the uncolonized plant extract also explains the ability of the plant without the involvement of microbes.

Conclusion

The data and evidence that we collected from our experiment suggest that *P. indica* has the ability to colonize the roots of *Cymbidium aloifolium*, a valuable orchid species, and promotes its growth and development. The bioactive compounds identified in colonized plant play a role in providing tolerance against drought and pathogen attack during field transfer. Therefore, *P. indica* can be used as a biological means for hardening the *in-vitro*-grown orchid plantlets to restore the ecology of the threatened orchid species.

Material and method

Establishment of in vitro *cultured C. aloifolium*

The mature green capsule of *C. aloifolium* was collected from the Makwanpur district of Nepal. The pod was surface sterilized. The seeds from the pod were then transferred to the well-standardized protocol for its growth on different strengths of Murashige and Skoog (MS) basal media for its germination.

Plant growth assay with P. indica

For the plant growth assay with *P. indica*, one-month-old plantlets were taken. The small plug of mycelium agar disc of *P. indica* was introduced near to the seedling on MS media flask supplemented with tryptophan. The growth and development activity of the seedling was monitored for 45 days. The five independent plantlets with three replicates were carried out with respect to the five independent *P. indica*-untreated seedling (control) to observe the significant colonization and seedling growth promotion activities of the *P. indica*. The plant growth assay was performed under aseptic conditions for 45 days under a 16 h photoperiod, 25 ± 2°C.

Histochemical analysis of the root sections

Ten random root sections from the fungus colonized plant was taken to investigate the pattern of colonization by fungus as per the method used in the previous work. The root sections were treated with 10% KOH solution and heated at 60°C for 15 mins followed by treatment of 1% HCl solution. Thereafter, the root sections were stained by 0.05% of trypan blue solution for 1 h followed by counterstaining with lacto phenol.[36] The root sections were observed under a light microscope.

Comparison of bioactive compound in colonized and uncolonized plant methanolic extract

The *in-vitro*-grown plantlets uncolonzsed as well as colonized by *P. indica* were taken after 45 days of their growth. The colonized and uncolonized (control) plantlets were separately ground with the help of mortar and pestle. Thereafter, it was suspended in High Performance Liquid Chromatography (HPLC) grade methanol (Fisher Scientific) for a day to extract bioactive compounds. The methanol extract was then further filtered by whatman filter paper Grade 1:11 µm and taken for chemical profiling by GC-MS technique.[37] The compounds were separated by GC technique and identified by MS. The instrument used for the experiment was GC-MS-QP2010 Ultra (Shimadzu Europa GmbH, Germany) instrument fitted with RTX-5MS (30 × 0.25 × 0.10 m) column. All the parameters were subjected. The initial and final temperatures of the instrument were set to 100°C and 250°C, respectively. Helium flow rate was 1 ml min^{-1} at 0.80 KV of ionization voltage. The splitless mode was set for the sample injection. Mass spectral scan range was set from 30 to 600 (m/z). The obtain chromatogram was then compared with the library of National Institute of Standard and Technology, NIST, US, to identify the bioactive metabolite.

Hardening and acclimatization of uncolonized and colonized plantlets

The transfer of *in-vitro*-grown plantlet to the natural environment is a very important step in which plantlets suffer high mortality. For orchids, different strategies were followed as previously described.[38–42] Pre-acclimatization of the *in-vitro*-grown plantlet was done in series of pre-acclimatization steps that were followed. The humidity inside the vessel of the *in-vitro* plantlets was reduced by making holes in the lid and left for 15 days.

For acclimatization, substrates such as cocopeat (P), charcoal (C), and gravel (G) were autoclaved and used. The different composition of the mixtures that were used was as follows.

Cocopeat (P): gravel (G): charcoal (C) = 2:2:1

The five plantlets colonized by *P. indica* and five uncoloniszd plantlets in three independent replicates were taken and growth was observed for 3 months in natural environmental conditions. In this process, the plantlets were transfered to the each sterilized pot containing the substrate.

Statistical analysis

The plant growth pattern in terms of shoot and root number as well as their length were calculated. The bar diagrams with standard mean and standard error were taken. One-way analysis of variance was done at the significant level of $P \leq 0.05$.

Acknowledgments

The research work was financially supported by University Grants Commission (UGC), Nepal. The authors are thankful to National Centre for Microbial Resource-National Centre for Cell Science, Pune, India, and Centre for Co-operation in Science & Technology among Developing Societies (CCSTDS), India, for facilitating GCMS instrumentation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed

Funding

This work was supported by the University Grants Commission, Nepal (NP) [PhD-72/73-S&T-Y-04].

Author's contribution

SS conceptualized the research work, performed *P. indica* and *C. aloifolium* interaction, GCMS analysis, histochemical microscopy of *P. indica*-colonized root section and prepared the manuscript. BBT did hardening and acclimatization as well as data recording. KC, PJ, and LS provided in-vitro *C. aloifolium* protocorms, BP and SP established the plant tissue culture protocol for C. *aloifolium*. AMS and AV provided *P. indica* for the experiment. BP overall supervised the entire research work. BP and SP did manuscript editing and revising.

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