Bioinoculants as a tool to improve total bacoside content in *Bacopa monnieri* L. (Pennell)

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Abstract - The aim of the present study was to evaluate the potential of five efficient bioinoculants [CRC1 (*Pseudomonas monteilii*, HQ995498/MTCC9796), CRC2 (*Cedecea davisae*, HQ995499/MTCC9797), CRC3 (*Cronobacter dublinensis* HQ995500/MTCC9798), CRC4 (*Advenella spp*., HQ995501/MTCC9799), CRC5 (*P. aeruginosa*, HQ995502/MTCC9800) useful in enhancing biomass and secondary metabolite content of *Bacopa monnieri*. The bioinoculants treated plants, over two harvests, produced significantly higher ($P \leq 0.05$) shade dried shoot biomass. The substantial improvements in herb yields were achieved with *P. monteilii* (CRC1) (17%) followed by *C. dublinensis* (CRC3) (12%), when compared to un-inoculated control plants during both harvests over two cropping seasons. The results of Fourier transform near infrared (FT-NIR) method showed a significant improvement (16-24%) in total bacoside content when plants were treated with bioinoculants. However, the higher concentration of bacoside content (24%) was estimated in the suckers inoculated with *P. monteilii* (CRC1). A significant improvement in nutrients (NPK) concentration was also noticed in bioinoculants treated plants compared to the un-inoculated ones.

Index terms - Bioinoculants, Bacopa, Bacoside content

1. INTRODUCTION

Bacopa monnieri L. Pennell (Indian Pennywort) (brahmi) is a prostrate and succulent herb, thick ascending branches with thyme leaved belongs to the family *Scrophulariaceae*. It is the second highest ranked herb (medicinal plants) used in traditional (*Ayurveda*) and modern systems of Indian medicine and popularly known as memory enhancer [5]. *B. monnieri* is commonly found and distributed in wet or marshy habitats and along the stream and river margins throughout India to approximately 1300 m elevation. The herb is reported to have the chemical constituent's brahmine and hespestine. Mannitol and saponins were reported later. It contains mainly two saponins namely bacoside A and B; which is considered as important active constituents are also known as "memory chemicals" [19, 23]. It has a great market demand due to its high medicinal properties i.e. analgesic [20], anticancer, antioxidant [36], adaptogenic [4], antidepressant [21], antianxiety, anti-epileptic effects [22]. It also possesses activity for antipyretic, epilepsy, insanity, and used in the treatment of asthma, hoarseness, water retention and blood cleaning [36, 37].

Plant growth promoting rhizobacteria (PGPRs) have gained considerable interest in recent years. Many rhizosphere colonizing bacteria especially PGPRs, including *Azotobacter*, *Azospirillum*, *Bacillus* and *Pseudomonas*, typically produce substances that stimulate plant growth or inhibit root pathogens [14, 8, 40]. A number of reports exists suggesting the use of bioinoculants such as plant growth promoting bacteria for enhancing growth and biomass yield of different medicinal and aromatic crops [24-29, 2] but only few reports suggest the usefulness of different microbes in bramhi [13,5].

Because of current public concerns about the harmful effects of agrochemicals, there is an increasing interest in improving the understanding of co-operative activities among rhizospheric plant beneficial microbial populations and how these might be applied to agriculture [11, 16]. Certain co-operative microbial activities can be exploited as a low-input biotechnology, and form a basis for a strategy to help sustainable, environmentally-friendly practices fundamental to the stability and productivity of both agricultural systems and natural ecosystems [12].

FT-NIR (Fourier transform near infrared) instrument is based on near-infrared spectroscopy (NIRS) which is a fast and economical analytical method used for both qualitative and quantitative analysis of various chemical substances [1]. In recent years, development associated with chemometrics towards application of near-infrared (NIR) measurements in medicinal and aromatic plants (MAPs) has emerged as a powerful technique. Through use of these techniques a quick quantitative estimation of bioactive molecules present in plant tissues can be achieved within short period of time [32].

The entire commercial requirement is met solely from the wild natural populations of *B. monnieri* resulting in its listing as a threatened plant [34]. Moreover, because of the heavy demand and short supply, it is the most adulterated species in *Ayurvedic* formulations. Their natural regeneration is hampered by death at two leaf stage and specific habitat requirement [6]. Therefore, there is need to develop efficient sustainable nursery technology for the healthy growth of *B. monnieri* which can lead to higher accumulation of its secondary metabolites content. A few bacterial isolate promoting the growth of *Bacopa* have been identified. The aim of the present investigation was to identify efficient bio-inoculants for the treatment of nursery cuttings subsequently transferred to pot con-

ditions for higher yield and bacoside content of *B. monnieri* utilizing FT-NIR technique used for quick estimation of total bacoside content.

2. MATERIALS AND METHODS

2.1Isolation, selection and molecular characterization of bacterial strains

The bacterial strains CRC1 and CRC5 were isolated from the rhizospheric soil [red sandy loam (Kandiustalf) in nature and had pH 6.3, and contained 0.37% organic carbon, available N (Alkali permanganate extractable) 218 kg ha^{-1}, available P (0.5 M $NaHCO_3$ extractable) 10.5 kg ha^{-1}, exchangeable K (NH_4OAc extractable) 230 kg ha^{-1}] of medicinal plant *Coleus forskohlii* and aromatic plant *Pogostemon cablin*, respectively. Rest of the bacterial strains (CRC2, CRC3 and CRC4) were isolated from vermicompost. Granular vermicompost had major nutrient concentration of 1.01% N, 0.78% K, 0.65% K and pH 7.02. Detailed proceditures about isolation, selection and 16S rDNA amplification and their sequence analysis of newly isolated strains CRC1, CRC2, CRC3, CRC4 and CRC5 was carried out as described earlier by Singh et al. [28] using the nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Further 16S rRNA gene sequence of strain CRC1, CRC2, CRC3, CRC4 and CRC5 was submitted to NCBI Genbank (HQ995498, HQ995499, HQ995500, HQ995501 and HQ995502 respectively).

2.2 Multiplication of bio inoculants

The bacterial cultures (CRC1, CRC4, and CRC5) were multiplied in nutrient broth and N-fixers/diazotrophs (CRC2 and CRC3) on Jensen's broth for 36 h at 210 rpm on an incubator shaker. The bacterial suspension was centrifuged at 8000 rpm for 10 min. The supernatants were discarded and the pellets containing bacterial cells were suspended in 500 mL of 100 mM phosphate buffer, pH 7.0. The CFU (colony forming unit) in this suspension for

bacterial strains was maintained between 1.5 and 2.8 × 10^8 mL^{-1}.

2.3 Planting materials

Healthy erect shoots with roots of *B. monnieri* cv. 'CIM Jagriti' were selected from the mother plants grown at research farm of CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow India. Cuttings of the plants consisting of 8-10 pairs of leaves having uniform length (6-8 cm) with roots were selected as nursery.

2.4 Nursery treatment and pot experiment

The selected nursery thoroughly washed in running tap water to avoid any adhering soil particles. Later on the nursery were dipped in their respective culture suspension (containing ≈2.0 × 10^8 bacterial cells mL^{-1}) in an individual Erlenmeyer flask for half an hour and nursery treated with sterile phosphate buffer (without culture) served as control. There were six treatments including control. The treated (CRC1, CRC2, CRC3, CRC4 and CRC5) and untreated (control) nursery were sown in earthen pots (25 cm in diameter and 30 cm long; two nursery pot^{-1}), holding approximately 7.5 kg potting mixture consisting of sterile soil and vermicompost (1:1/10, v/v), replicated four times in completely randomized design (CRD). Vermicompost was added as a nutrient supplement for plant growth irrespective of the treatments. The respective culture suspension (5 mL pot^{-1}) was also poured in each treatment. The experiments were performed under glasshouse conditions with minimum and maximum temperature of 25 and 34˚C, respectively, a relative humidity of 60–70%, and an approximate 16:8 (day/night) photoperiod. The soil used in this experiment was a sandy loam (Ustifluvent) with pH 7.35, EC 0.38 dSm^{-1}, 3.35 g kg^{-1} organic carbon, 191 kg ha^{-1} available N (alkaline permanganate extractable), 12.9 kg ha^{-1} available P (0.50 M

$NaHCO_3$ extractable), and 102 kg ha^{-1} available K (1 N NH_4OAc extractable). The vermicompost used in the pot experiment was produced from mixture of distillation waste (plant-spent de-oiled herb) of menthol mint (*Mentha arvensis*) in a vermicomposting unit for 90 days using adult clitellate *Eudrilius eugineae*, epigeic species of earthworms [27, 28]. The vermicompost contained 1.85% N, 0.85% P and 0.78% K. Watering was carried out with fine rose can. The plants were harvested two times during each cropping season. The first harvesting was done after 90 days of transplanting and the second harvest after 60 days of first harvest. Fresh herb biomass was recorded from each pot. To estimate total bacoside content fresh herb was thoroughly cleaned with water. After the cleaning, the herb was spread thinly on clean gunny bags in shade and allowed to air dry about 8-10 days. The material was turned over frequently to avoid fungal growth. The shade dried material was pulverized into fine powder form. Fresh shoot biomass sample (25g) was dried in a hot air oven at 80˚C for 24 h to determine the moisture content and nutrient concentration. Nutrient concentration (NPK) in dried shoot was determined by Jackson [10]. Initial soil samples were analyzed for total organic carbon (TOC) (%), available N (kg ha^{-1}), available P (Olsen's P_2O_5, kg ha^{-1}) and available K (exchangeable K_2O, kg ha^{-1}) following Jackson [10]. The rhizospheric soil samples from each replicated treatments were collected at the time of harvesting and pooled soil samples from each treatment were employed to estimate bioinoculants population. Pseudomonads, N–fixer and P solubilizers populations [colony forming unit (CFU) g^{-1}] in the root zone soil was determined by serial dilution (10^{-2}folds) with 0.85% saline solution using King's B medium [41] , Jensen's medium and P solubilizing bacterium zone formation and cultivation medium [18] in triplicate, respectively. King's medium was supplemented with different concentration of antibiotics (CRC1: 25µg

mL^{-1} medium rifampicin, CRC5: 10µg mL^{-1} medium Kanamycin), Jensen's medium was supplemented with different concentration of antibiotics (CRC2: 10 µg mL^{-1} medium streptomycin sulphate, CRC3: 30µg mL^{-1} medium rifampicin) and Pikovskaya's medium was supplemented 100 µg mL^{-1} medium streptomycin sulphate for estimating selective population of pseudomonads, P solubilizers and N-fixers found to be tolerant to particular antibiotics and concentrations.

2.5 Estimation of total bacoside content

An in-house developed Fourier transform near infrared (FT- NIR) method, which was and well validated with the results from HPLC analysis, is used for the analysis of total bacosides in the *B. monnieri*, plant materials [1]. The method is faster than the traditionally used chromatographic methods and it does not have the problems related to sample preparations, use of solvents which are expensive as well as hazardous to both environment and the human, and too much time required for the traditional measurements. The method we developed and used here is comparable to routine HPLC method and about 40 times faster than the HPLC based method [1].

For the screening of the samples, 2mg of finely powdered *B. monnieri* plant samples were used. Each plant sample was subdivided into 40 sub-samples of 50µg in weight. Every sub-sample was subjected for FT-NIR analysis 8 times. After each analysis the sub-sample was remixed before taking a new reading. Thus, from one sample of 2 mg of *B. monnieri*, a total of 320 FT-NIR spectra were taken for the analysis [1].

2.6 Fourier transform near infrared (FT- NIR) measurements

FT-NIR absorbance spectra from $10000cm^{-1}$ to $4000cm^{-1}$ at a resolution of $2cm^{-1}$ were recorded on Antaris II analyzer fitted with InGaAs detector (Thermo Fish-

er Scientific, USA) in diffuse reflectance mode for each sample. Each spectrum was the average of 64 scans. Background measurements were taken before every spectral measurement. The measurements were pre-processed and analyzed using TQ Analyst Software (Thermo Fisher Scientific, USA). The Partial least squares model for the determination of total bacoside was developed using TQ Analyst.

2.7 Statistical analysis

The collected data were subjected to statistical analysis for analysis of variance method (ANOVA), suitable to completely randomized design (CRD) for pot experiment with the help of software ASSISTAT Version 7.6 beta (2012). Significant differences among treatments were based on the *F*-test in ANOVA and means were calculated using Duncan's multiple range test under a significance level of $P \leq 0.05$. There were two trials conducted for pot experiments. The experimental data of two trials had a similar variance value; hence the data were combined for further analysis. The results and discussion are based on the average of the trials of each experiment. The standard error (SE) of means in vertical bar charts was computed using Sigma Plot 11.

3. RESULTS AND DISCUSSION

3.1 Molecular/16S rRNA gene sequence analysis-based identification of selected bacteria

The BLAST analysis of the 16S rRNA gene sequence from the CRC1/CRC5, CRC2, CRC3 and CRC4 isolate revealed that the bacteria belong to the genus *Pseudomonas, Cedecea, Cronobacter* and *Advenella*, respectively and their relation to other related species is given in Table 1. Phylogenetic analysis showed that isolate CRC1, CRC2, CRC3, CRC4, and CRC5 has a maximum similarity with *P. monteilii* strain WAPP53 (accession no. FJ905913), *C. davisae* str. NBRC 105,702

(accession no. AB682275), *C. dublinensis* str. G3977 (accession no. HQ880415), *Advenella spp*. Str. SS-2009-PON8 (accession no. FN646615) and *P. aeruginosa* str. BP C1 (accession no. JQ796859), respectively; therefore isolate CRC1, CRC2, CRC3, CRC4, and CRC5 were designated as *P. monteilii, C. davisae*, *C. dublinensis*, *Advenella* spp., and *P. aeruginosa* respectively. The 16S sequence of isolate CRC1, CRC2, CRC3, CRC4, and CRC5 was submitted to Genbank (NCBI) under the Accession number HQ995498, HQ995499, HQ995500, HQ995501 and HQ995502, respectively. This identification of the isolates was later confirmed by CSIR-IMTECH (Institute of Microbial Technology), Chandigarh, India, and the isolates CRC1, CRC2, CRC3, CRC4 and CRC5 are deposited with Microbial Type Culture Collection (MTCC), Chandigarh, India with MTCC nos. 9796, 9797, 9798, 9799 and 9800 respectively (Table 1).

Table 1 Homology search of bacterial isolates

Isolate	Identification	GenBank accession number	Similar organism	GenBank accession number	Sequence Similarity (%)
CRC1	*Pseudomonas monteilii*	HQ995498	*Pseudomonas monteilii*	FJ905913	99
CRC2	*Cedecea davisae*	HQ995499	*Cedecea davisae*	AB682275	97
CRC3	*Cronobacter dublinensis*	HQ995500	*Cronobacter dublinensis*	HQ880415	99
CRC4	*Advenella* sp.	HQ995501	*Advenella sp.*	FN646615	99
CRC5	*Pseudomonas aeruginosa*	HQ995502	*Pseudomonas aeruginosa*	JQ796859	99

3.2 Effect of bioinoculants on total herb yield over two harvests

The success of bioinoculants depends on the selection of potential bioinoculants/antagonist, method and mode of application, the right environment and other

factors [24]. Generally, the objective of nursery inoculation is not to achieve a growth response, but rather to establish a strong relationship with the plant so that it can be effectively transferred to the pot/field [33, 9, 3]. Inoculation with bio-inoculants at very early stages has been found to result in higher crop uniformity, reduce transplant mortality [39] and higher yields after transplanting to the pot/field [15, 38]. Since in most soils the indigenous population bioinoculants are present, the pre-inoculation of cuttings at nursery stage, provides the introduced bacterial/ fungal strains a special advantage over the indigenous bacterial/fungal strains after transplanting in pot/field [7, 31].

All the bio-inoculants treated plants, over two harvests, produced significantly higher shade dried herb biomass (Table 2); CRC1 being most effective yielding higher (18.4%) herb followed by CRC3 (12.9%) compared to un-inoculated control plants over two harvest (Table 2); similar trends were observed during both harvests (Table 2). Singh et al. [27, 28] observed that in-oculation of *P. monteilii* Strain CRC1 in *C. forskohlii*, and *O. basilicum* plants signifi-cantly improved the root and shoot bio-mass. In another study of Singh et al. [26, 27, 28] plant growth promoting bacteria and AM fungi significantly improved the shade dried herb biomass of patchouli (*P. cablin*). Tiwari et al. [35] reported that in-oculation of endophytic bioinoculant *B. subtilis* significantly improved the growth and fresh biomass yield of *O. sanctum.* In our recent study an efficient PGPR *Micro-bacterium* sp. (strain SUCR140) improved growth and yields of maize plants com-pared to control [30].

Table 2 Effect of bioinoculants on dry matter yield of *Bacopa*

Treatment	*Dry herb yield (g pot^{-1}) I Harvest	*Dry herb yield (g pot^{-1}) II Harvest	*Total dry herb yield (g pot^{-1})
Control	65.29 c	37.03c	102.33d
CRC1	76.89a	44.22a	121.11a
CRC2	70.75b	40.59b	111.35c
CRC3	73.39ab	42.11b	115.50b
CRC4	71.20b	39.91b	111.12c
CRC5	69.76b	41.37b	111.14c

Values in each column followed by different letters are significantly different at $P \leq 0.05$;
CRC1= *Pseudomonas monteilii;* CRC2= *Cedecea davisae;* CRC3= *Cronobacter dublinensis;* CRC4= *Advenella sp.;* CRC5= *Pseudomonas aeruginosa;* *= values are mean of two trials.

3.3 Effect of bioinoculants on total bacoside content

The FT-NIR spectra showed broad bands of overlapping absorption bands arised from harmonics and combinations of fundamental molecular vibrations. During the analysis of the secondary derivative spectra of the raw spectra obtained from the bacopa samples, it was found that at three spectral regions i.e. 7308 – 6800 cm^{-1}, 8720 – 8120 cm^{-1} and 4546 – 4420 cm^{-1} the variations in absorbance values have significantly influenced by the total baccosides content in the shade dried powdered plant samples. One of such variations is shown in (Fig. 1A). The total bacoside content values obtained from the model are given in Fig. 1B. Bioinoculant treated nursery significantly improved (16-24%) (Fig. 1B) the total bacoside content over control whereas the maximum enhancement (24%) was noticed with CRC1 (Fig. 1B). The similar trends were observed in medicinal plant like *Coleus forskohlii* and *Artemisia annua* where inoculation with efficient bioinoculants (pseudomonads, AM fungi and N-fixers) significantly improved the forskolin and arte-

misinin content [26, 27, 2]. In one of our recent study Singh et al. [27] studied that the inoculation/seed treatment of *Ocimum basilicum* with *P. monteilii* strain CRC1 significantly increased in the concentration of linalool and caryophyllene of basil essential oil.

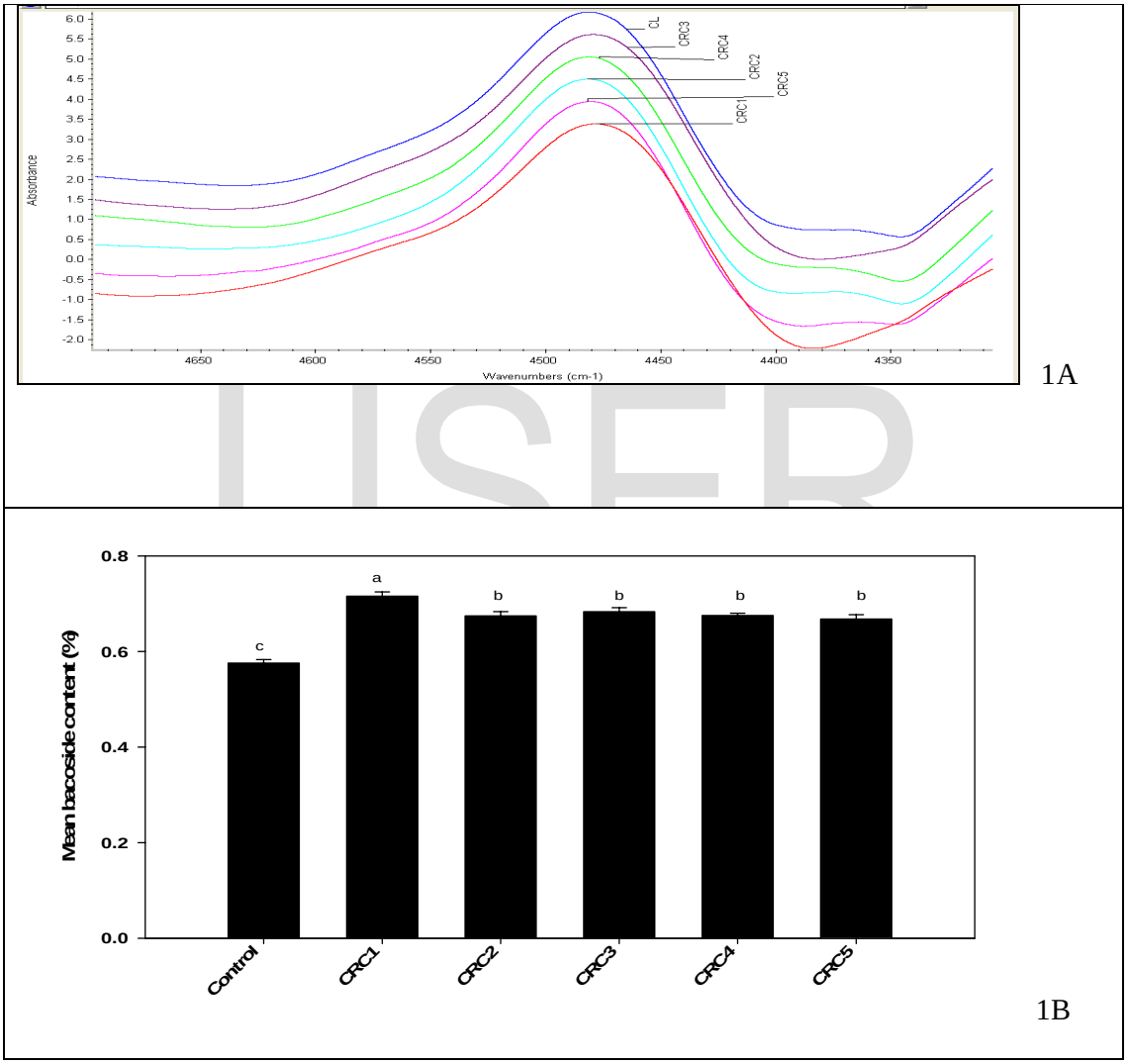

Fig.1 Fourier transform near infrared (FT- NIR) analysis (A) of *Bacopa* smaples treated with bioinoculants for total bacoside content (B); *Error bars* in Fig.1B shown as standard error of mean(SE). Different letters above the error bars indicate a significant difference at $P \leq 0.05$; CRC1= *Pseudomonas monteilii;* CRC2 = *Cedecea davisae;* CRC3= *Cronobacter dublinensis;* CRC4= *Advenella sp.;* CRC5= *Pseudomonas aeruginosa.*

3.4 Effect of bioinoculants on nutrient concentration and rhizospheric bioinoculants populations at harvesting stage

All the bioinoculants treated nursery significantly improved the N and K content (16-38% and 17-81%, respectively) compared to un-inoculated control (Table 3), however, the maximum increase in N (38%) and K (81%) content was noticed with CRC3 and CRC1 (Table 3), respectively. CRC1 and CRC3 significantly increased (26 and 18% respectively) P content in dried herb biomass compared to un-inoculated control (Table 3). In our earlier and recent studies it was clearly demonstrated that inoculation with PGPRs significantly improved the concentrations and uptake of major nutrients in root and shoot biomass [24, 26, 28]. The other study [17] also provided a clear evidence for improving nutrients content by the inoculation with PGPRs.

Table 3 Effect of bioinoculants on NPK concentration of *Bacopa*

Treatment	*N (%)	*P (%)	*K (%)
Control	1.04d	0.24bc	1.05f
CRC1	1.28b	0.31a	1.88a
CRC2	1.27b	0.24c	1.49c
CRC3	1.43a	0.29a	1.69b
CRC4	1.19c	0.26b	1.22e
CRC5	1.27b	0.25bc	1.43d

Values in each column followed by different letters are significantly different at $P \leq 0.05$;
CRC1= *Pseudomonas monteilii;* CRC2= *Cedecea davisae;* CRC3= *Cronobacter dublinensis;* CRC4= *Advenella sp.;* CRC5= *Pseudomonas aeruginosa;* *= values are mean of two trials.

The microbial population (CFU g^{-1} soil) of fluorescent pseudomonads [CRC1 (4.5×10^5) and CRC5 (4.5×10^5)], N-fixers [CRC2 (4.7×10^4) and CRC3 (6.1×10^4)] and P solubilizers [CRC4 (1.8×10^6) were maintained at substantial level at the time of harvesting. In general, the population of a particular bioinoculant was increased in the rhizosphere of the plants inoculated with the same organism. The similar trends were observed when *C. forskohlii* and *O. basilicum* plants were inoculated with efficient bioinoculants [24, 25, 26, 27].

4. CONCLUSION

The present studies undoubtedly indicated that native efficient bioinoculants used for nursery treatment could be successfully transferred to pot conditions resulting in improved biological management of nutrients leading to yield improvement which may be further translated to field conditions to improve bacoside content in sustainability manner. The usage of FT-NIR provided a valuable platform for quick economical estimation of total bacoside content. So this bioinoculants mediated technology could be a better option for sustainable agriculture especially in medicinal plants where the use of chemicals is restricted because of health and residue considerations. The implementation of this model to other medicinal plants will provide an edge in development of safe and greener technology.

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