Bioinoculants for growth enhancement of aromatic plants: *Artemisia pallens* and *Origanum majorana*

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Abstract

The use of fertilizers is a common practice to improve soil fertility and increase crop yields. Chemical fertilizers have played a significant role in increasing crop yields, but their widespread use has led to environmental degradation and soil depletion. The continuing challenges in agro-ecosystem and environment require more sustainable solutions than chemical fertilizers. In recent years, there has been a growing interest in the use of biofertilizers as a sustainable alternative to chemical fertilizers. Biofertilizers are natural substances that improve soil fertility, modify soil microbiota and enhance plant growth. Cyanobacteria are one of the well-known bio inoculants for paddy crops. However, the use of cyanobacteria for other plants has not been very well documented. We screened certain cyanobacteria isolated from different natural environments as biofertilizers for certain aromatic plants.

Artemisia pallens and Origanum majorana are two plants with aromatic compound productions. The aim of this study was to screen for enhancement in the growth and propagation of the plants for their yield using bio inoculants. Further air-dried, powdered plant materials of the aerial parts of A pallens and O. majorana were extracted using solvents ranging in polarity from non-polar (n-hexane), semi-polar (chloroform) and polar (acetone, methanol). These extracts were tested for antioxidant activity, several plant extracts showed antioxidant activity, with water exhibiting the greatest levels.

The purpose of the experiment was to investigate the effects of bioinoculants on the growth of the test plant. Our observation showed that all the tested cyanobacterial strains i.e. KM1, KM2 and VM1, VM2, the bioinoculants, enhanced plant growth to various levels. KM1 and KM2 exhibited a greater ability to increase plant growth. This demonstrates that Artemisia's and Origanum's growth and herbage output may be successfully enhanced by the use of cyanobacterial bioinoculants.

Keywords: *Artemisia pallens*, *Origanum majorana*, artemisinin, cyanobacteria, antioxidant activity, phytochemical analysis.

Introduction

The use of biofertilizers has been shown to have beneficial effects on soil fertility and crop productivity[4]. Biofertilizers are eco-friendly and sustainable alternatives to chemical fertilizers that contain living microorganisms such as bacteria, fungi and algae which help improve soil fertility and plant growth[14]. Chemical fertilizers can be harmful to the environment when abused and can also be costly[6]. Therefore, there has been increasing interest in finding eco-friendly and sustainable alternatives to chemical fertilizers. The use of biofertilizers has been gaining attention worldwide due to their positive effects on soil fertility, crop productivity and environmental sustainability[17].

Cyanobacteria, also called blue-green algae, evolved very early in the history of life[26]. Present cyanobacteria show characteristics of both eubacteria and higher plants[25,36]. They are simple organisms either unicellular or filamentous and often grow in large colonies[30]. Cyanobacteria are composed of cells exhibiting a broad range of shapes and sizes[28]. Overall, 150 genera have been identified so far[13]. A natural population of cyanobacteria exists in the majority of paddy soils, offering a free source of nitrogen fixation[35]. Cyanobacteria may absorb ammonia either as ammonium (NH_4) or by passive diffusion using a particular absorption method[18].

Cyanobacteria are usually composed of two types of cells namely heterocysts and vegetative cells[11]. The use of cyanobacteria as a biofertilizer has been shown to enhance plant growth and yield in various crop species including aromatic plants such as lavender, rosemary and thyme[16].

Along with food crops, a significant portion of the flora is made up of medicinal and aromatic plants which serve as the source of raw materials for the pharmacological, aesthetic and medicinal industries[15]. The foundation of traditional medicine in many nations has traditionally been aromatic plants and culinary herbs[24]. Recent studies have shown that the use of antioxidant-rich species such as cloves, cinnamon, oregano, rosemary, ginger, black pepper, paprika and garlic, added to meat during cooking resulted in the reduction of meat oxidation and the *in vivo* reduction of plasma and urine malonaldehyde concentrations, offering scientific proof of the health advantages of including antioxidant spices in rich, fatty foods[9,34]. These spices possess significant antioxidant activity and can be used to prevent oxidative damage[29].

Furthermore, the medicinal properties of plants are often attributed to their bioactive compounds such as phenols and flavonoids[2].

Artemisia pallens and *Origanum majorana* (Fig. 1) are two aromatic plants widely used in traditional medicine, perfumery and culinary arts[1,23]. *Artemisia pallens*, commonly known as Davana, is a drought-tolerant herb that can grow well in poor soils and harsh environments[32]. It has been used for centuries in traditional medicine to treat various ailments such as respiratory disorders, digestive issues and menstrual problems[5,7]. The essential oil extracted from the plant is rich in sesquiterpenes which give it a unique aroma and medicinal properties[20].

Origanum majorana, commonly known as sweet marjoram, is a herbaceous perennial plant that has been used for centuries in traditional medicine to treat various ailments including digestive disorders and respiratory infections[33]. Its essential oil is rich in monoterpene hydrocarbons and oxygenated monoterpenes, which give it a unique aroma and a range of therapeutic properties[27].

On the other hand, *Origanum majorana*, commonly known as sweet marjoram, is a popular culinary herb that has been used in Mediterranean cuisine for centuries[31]. It is a perennial plant that belongs to the mint family and is known for its aromatic leaves that are rich in essential oils[22]. The essential oil extracted from the plant has been found to possess various medicinal properties including antibacterial, antifungal and anti-inflammatory effects[8]. Additionally, it has been reported to have antioxidant and neuroprotective effects[10].

Overall, *Artemisia pallens* and *Origanum majorana* are versatile plants with a wide range of uses in various industries. Their potentials for sustainable agriculture and medicinal and aromatic properties make them promising crops for cultivation[21,37].

Material and Methods

Sample Collection: Random water samples were isolated from Korangi Mangrove Forest (16°30′ 52.9″ N 82°14′80.4″ E) located in Kakinada, Andhra Pradesh and from the industrial mangrove area in Visakhapatnam, Andhra Pradesh. The collected samples were named KM1, KM2 and VM1, VM2 respectively.

Isolation and Growth of Cyanobacterial Cultures: The cyanobacterial samples collected as above were separated into individual strains by repeated shaking and subculturing from single colonies. The cultures were grown for 14 days under artificial light in 250 mL flasks using BG-11 media. This work focuses on using cyanobacterial extracts as a biofertilizer to nourish plants directly. For that, two rapidly developing heterocystous cyanobacterial cultures were taken from the previously inoculated samples and were again reinoculated for 15 days. The optical density of the culture was measured using a spectrophotometer at 660 nm.

Extraction Method: After 4 weeks of incubation, the cyanobacteria cultures were harvested. Every day, 1 L of culture was withdrawn and centrifuged at 5000 rpm for 15 minutes and, distilled water was used to wash the cells. The cyanobacteria were homogenized in regular saline to create the cell suspension (Fig. 2). A suspension comprising 500 mL of regular saline or water and 5.0 g of fresh cyanobacteria material is regarded as a 1% extract. The extract was filtered.

Fig. 1: Crops of *Artemisia annua.* and *Origanum majorana*

Fig. 2: Cyanobacteria inoculation in BG11 media. (a) shows culture without homogenization and (b) shows homogenized culture

Plant Material: The plants (*Artemisia pallens* and, *Origanum majorana*) used for this study were collected from a nursery located in Visakhapatnam, Andhra Pradesh.

Experimental conditions for pot experiments: To assess the impact of the test cyanobacterial strains on the yield of *Artemisia pallens* and *Origanum majorana*, a pot culture experiment was designed. The plants were grown in sterile soil. For 30 days, three plants of each were raised in 3-litre pots (14 cm in diameter) which contained 60% coco peat, 25% sand and 15% regular soil. In the morning, suspensions of chosen cyanobacteria in the log phase (4–15 days old) were introduced to the potted plants. As inoculations, 100 ml of a 1% cyanobacterial solution containing 1 g of cyanobacterial cells was employed. One such bacterization was given every two weeks.

Plant growth parameters: The parameters employed to assay plant growth were plant height, root length, dry and fresh weights, leaf number, leaf area and the number of branches. In addition to growth, the quantity of the essential oils extracted from these plants was also analyzed

Preparation of cell extracts: The cyanobacterial cultures were harvested after one month of growth by centrifugation at 5000 rpm for 15 minutes. In each case, the algal pellet was collected, weighted and used for extraction of antibacterial agents. One gram of dried powder of each of the three algal pellets was extracted in 10 ml, either with chloroform, methanol, or water to get extract compounds with increasing polarity by shaking overnight for complete extraction. The extract was filtered and stored at -20ºC for further studies. The concentration used was 1 mg/ml by dissolving in the same solvent used for extraction and was assayed for antibacterial activity.

Chlorophyll Analysis: One gram of *Artemisia pallens* or *Origanum majorana* leaves was finely chopped and gently combined in a cleaned pestle and mortar. This homogenized leaf material was then mixed with 5 ml of 80% acetone. The materials underwent gentler grinding. After that, the sample spent 4 hours in a 40º C refrigerator. The material was then centrifuged for 5 minutes at 3000 rpm. Using 645 and 663 nm wavelengths in opposition to the solvent, a spectrophotometer assessed the colour absorbance of the solution. As a blank, acetone (80%) was utilized.

Formula:
Chl a= 11.75×A663 – 2.35×A645 ×V/1000×W
Chl b=18.61×A645 – 3.96× A663 ×V/1000×W
[8.02×A663 + 20.20xA645] ×V/1000×W
Chlorophyll a + b

where Chla and Chlb are chlorophyll a and chlorophyll b and A is absorbance.

Antibiotic Activity: Plant material that had been air-dried and powdered (3 g) was extracted using polar (distilled water), semi-polar (chloroform) and polar (methanol) solvents over the course of 24 hours. In order to produce crude extracts, the solvent was collected. Two bacterial strains (*Escherichia coli* ATCC - 11246 and *Staphylococcus aureus* ATCC - 6538 P.) were the subject of antimicrobial investigations. The bacteria used were collected from agar slants that were less than 30 days old. The bacteria were cultivated on nutrient broth and cultured for 24 hours at 37°C.

Antibacterial activities were determined by the paper disk (5 mm) diffusion method. 50 mg samples of each extract were dissolved in the appropriate solvents (2 ml). 50 µl of these solvent extracts (8 mg/disc) were impregnated into sterile 5 mm diameter filter paper discs. On the surface of the solid medium on plates, the test organisms (0.1 ml) were inoculated and spread using a sterile spreader. Before putting the extract-impregnated paper discs on the agar plates, the test organism-inoculated plates were incubated for one hour. The sterile discs that had been impregnated with various extracts were then put on agar plates. After being incubated for 24 hours at 37 °C, the bacterial plates were all examined for zones of growth or inhibition and the widths of these zones were determined in millimeters. All testing was conducted in sterile environments maintained throughout the procedure.

Antioxidant Activity: The four different solvent extracts (chloroform, hexane, ethanol and water) were prepared from *Artemisia pallens* and *Origanum majorana* separately. The antioxidant activity of the extracts was measured using the DPPH assay. Briefly, 3 ml of DPPH solution was added to 2 ml of each extract at different concentrations (0.1, 0.3 and 0.5 ml). DPPH is a stable free radical that reacts with antioxidants, resulting in a color change from purple to yellow which can be measured spectrophotometrically. The mixture was incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 517 nm. The antioxidant activity was calculated as a percentage of Inhibition by using the following formula:

% Inhibition = [(Control absorbance – Test absorbance) / Control absorbance] x 100

Results and Discussion

Growing *Artemisia pallens* and *Origanum majorana* in pots with cyanobacterial extracts helped in plant growth. Table 1 shows a comparison between treated and control plants revealing a considerable change. Root length, plant height and fresh, newly emerging leaves from the shoot nodes were the plant metrics utilized to gauge growth. The outcomes showed a substantial change in the majority of measuring parameters between the control and various plants treated with cyanobacterial extracts. Differences between the plants treated with the two cyanobacterial strains were also noted. For instance, shoot length showed the least variation between treatments and controls, but leaf number demonstrated the highest difference.

In the pot experiments of the study, it was shown that inoculation with certain cyanobacterial isolates considerably encouraged the growth of leaves and plants in pots. The improvement in total plant biomass and the rise in yield were positively correlated. Extracts of cyanobacteria strains of KM1 and VM1 were used as an inoculum for *Artemisia pallens* and the extracts of KM2 and VM2 were used as an inoculum for *Origanum majorana*. Treatment of *Artemisia pallens* and *Origanum majorana* with cyanobacterial extracts caused various alterations in the morphology of the treated plants as compared to the controls such as change in the density and size of the roots stem and leaves were markedly different.

Treatment details: The soil of treated pots was supplemented with 1% algal extract (5.0 g fresh algal material in 500 mL of distilled water).

Plant treated by cyanobacteria isolated from Vizag VM1
Plant treated by cyanobacteria isolated from Vizag VM2
Plant treated by cyanobacteria isolated from Kakinada KM1
Plant-treated by cyanobacteria isolated from Kakinada KM2
Plant inoculated by distilled water (control plant).

The results of table 2 demonstrated that the tested microorganisms were moderately sensitive to plant extract samples when they were tested against antibiotic standard discs. *Escherichia coli* and *Staphylococcus sp.* were evaluated and according to the zone of inhibition, the plant extracts containing methanol and chloroform were able to inhibit bacterial growth moderately. However, this inhibition was quite less compared to tested standard antibiotics in table 2. *Staphylococcus aureus* was found to be inhibited by the majority of the cyanobacterial strains with inhibition zones of 3-5mm.

A comparable observation in the natural environment has been obtained earlier. A noticeable decrease of gram-positive bacteria in lakes during the occurrence of cyanobacterial water blooms has been reported by Anburaj et al[3] which could be explained to be caused by the release of some antibacterial compounds by the bloom formers.

Table 1
Growth Analysis of *A. pallens* and *O. majorana*

Plants	***Artemisia pallens***			***Origanum majorana***		
Treatment with bioinoculant	Control	VM1	KM1	Control	VM2	KM2
Leaf number (after 30 days)	21	30	34	76	154	216
Plant height (cm)	28	28	28	7	5.5	5.5
Number of branches	7	10	14	11	17	24

Table 2
Antimicrobial activity of plant extracts

Test organism	**Type**	***Artemisia pallens***			***Origanum majorana***			***Standard Zones***
	Gram	Methanol Extract	Chloroform Extract	Distilled Water	Methanol Extract	Chloroform Extract	Distilled Water	Ampicillin
Staphylococcus aureus	positive	3mm	5mm	1mm	-	-	-	20-28mm
Escherichia coli	negative	2mm	3mm	-	-	-	-	15-25mm

Table 3
Antioxidant activity of plant extracts

Solvent extracts	**Concentration**	**Antioxidant activity**	
		Artemisia pallens	***Origanum majorana***
Chloroform extract	0.1	8.55 ± 0.66	24.84 ± 0.59
	0.3	2.51 ± 1.32	16.29 ± 0.19
	0.5	0.54 ± 0.41	3.70 ± 0.58
Hexane extract	0.1	39.28 ± 0.99	13.57 ± 0.25
	0.3	13.32 ± 1.55	12.15 ± 0.85
	0.5	1.77± 0.68	2.95 ± 0.82
Ethanol extract	0.1	36.79 ± 1.61	80.69 ± 0.70
	0.3	25.34 ± 0.62	40.52 ± 0.62
	0.5	5.56 ± 1.29	35.09 ± 1.29
Water extract	0.1	54.95 ± 0.62	40.36 ± 0.71
	0.3	35.29 ± 0.60	36.53 ± 1.09
	0.5	27.09 ± 0.76	23.85 ± 1.74

The antioxidant activities of *Artemisia pallens* and *Origanum majorana* extracts prepared in ethanol, chloroform, hexane and water at varying concentrations were examined. A stable free radical called DPPH may take an electron or a hydrogen ion to transform into a stable diamagnetic molecule. Antioxidants' capacity to donate hydrogen is regarded to be what causes them to be effective in scavenging DPPH radicals. The reduction in DPPH radicals' absorbance at 537 nm brought on by antioxidants was used to gauge their capacity for reduction. Figure 4 shows the methanol extract of *Artemisia pallens* ability to scavenge DPPH free radicals. Table 3 lists the DPPH free radical scavenging activity values for water extracts, ethanol extracts, hexane extracts and chloroform extracts. Lower quantities of hexane extract and ethanol showed the best antioxidant efficacy. But as extract concentration increased, antioxidant activity decreased.

The results of the DPPH assay showed that all extracts from both plants had antioxidant activity. The water extract of *Artemisia pallens* showed the highest antioxidant activity among all the extracts, with a maximum percentage of radical scavenging activity of 54.95% at a concentration of 0.1 ml. The water extract of *Origanum majorana* showed a maximum percentage of radical scavenging activity of 40.36% at a concentration of 0.1 ml. The ethanol and hexane extracts also showed antioxidant activity while the chloroform extracts showed the lowest antioxidant activity.

Table 4 shows the total chlorophyll content of the plant extracts. *Origanum majorana* showed a higher content of chlorophyll a (7.51 µg/g) and chlorophyll b (4.23 µg/g) than *Artemisia pallens* (5.95 µg/g and 2.09 µg/g respectively). The total chlorophyll content of *Origanum majorana* (11.74 µg/g) was also higher than that of *Artemisia pallens* (8.04 µg/g). As both plants require low temperatures and humid conditions for growth, the chlorophyll pigments were low[12,19].

Fig. 3: Comparison between control and treated plants in growth parameters

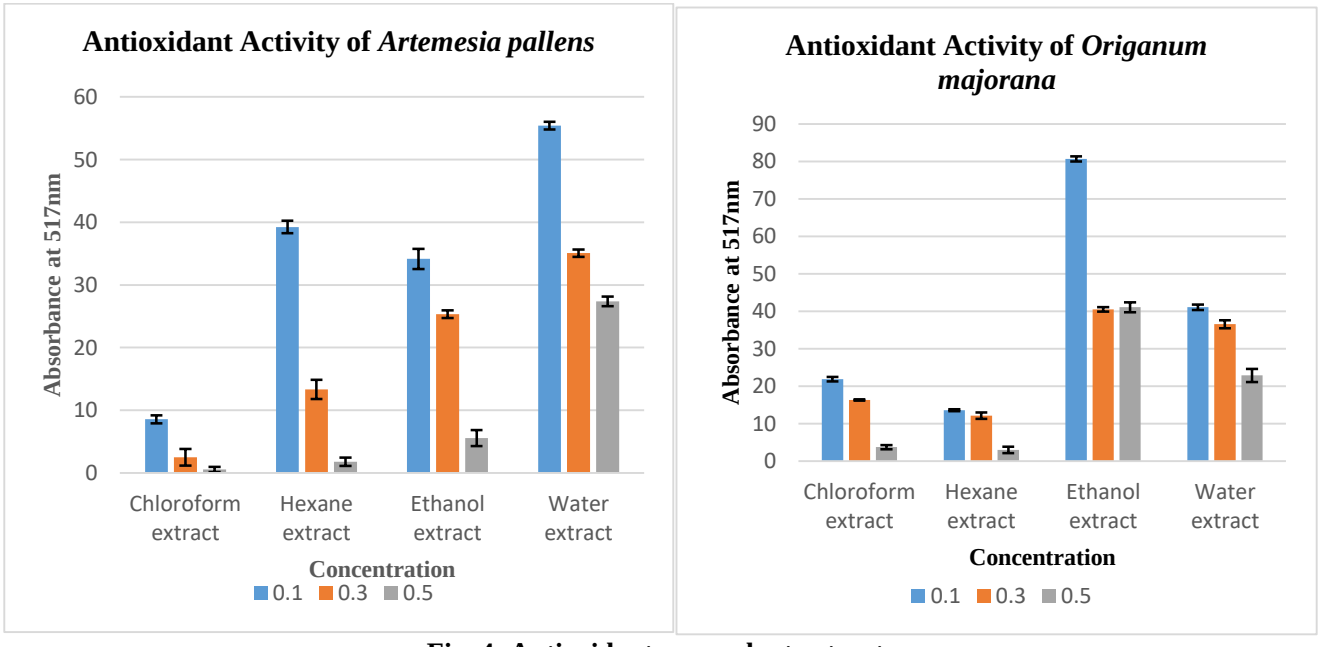

Fig. 4: Antioxidant assay plant extracts

Table 4
Chlorophyll Analysis of plant extracts

Plant	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Artemisia pallens	5.95	2.09	8.04
Origanum majorana	7.51	4.23	11.74

Conclusion

Cyanobacteria were used as bioinoculant to enhance plant growth and biomass. They also improved the development of lateral shoot buds. The cyanobacterial strains (KM1 and KM2) were proven to provide a better yield when compared to the other two cyanobacterial bioinoculants. When treated with cyanobacteria, the findings indicated a sharp rise in plant laterals which eventually helped in improving the output. Natural antioxidant and weak antibacterial properties are present in both plants. The results of this study suggest that the plant extracts tested possess significant antioxidant activity which could be attributed to the presence of phenolic and flavonoid compounds.

Both *Artemisia pallens* and *Origanum majorana* have antioxidant activity, with the water extract showing the highest activity. These findings suggest that these plants have the potential to be used as natural antioxidants in various applications including the food and pharmaceutical industries. The higher chlorophyll content observed in *Origanum majorana* could potentially contribute to its superior growth and photosynthetic efficiency compared to *Artemisia pallens*. *Artemisia pallens* showed antibiotic action with staphylococcus species, according to experimental research. Staphylococcus species-related infections can be prevented with artemisia. Further studies are needed to investigate the specific antioxidant compounds present in these plants and their mechanisms of action.

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