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Effect of biofertilizers and humic acid on vegetative growth parameters in Sat Kara *(Citrus macroptera)* under soil media conditions

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ABSTRACT

An investigation on effect of biofertilizers and humic acid on vegetative growth parameters in Citrus macroptera seedlings under soil media conditions was studied during the period 2022–2023. Result after 2 months of transplanting of seedlings in the soil media, it was revealed that application of Humic acid (T_8) gave maximum plant height (11.63 cm); number of leaves/plant (7.33); leaf area/plant (5.63 cm^2); root length (14.37 cm); number of fibrous root (20); root diameter (1.65 mm); shoot: root ratio (3.98); fresh weight of shoot & root (0.89 g & 0.20 g); dry weight of shoot& root (0.234 g & 0.059 g); vigour index-1 & 2 (1123.33, 22.76); survival % in soil media (96.29%); chlorophyll-a, b & total chlorophyll (1.36 mg/g, 0.69 mg/g, 2.05 mg/g).Thus, the experiment concluded that the treatment T_8- Humic acid, which produced the most favourable results among all treatments, is most promising with results to meet the objectives of the experiment.

Keywords: Biofertilizers, *Citrus macroptera*, humic acid, hydroponics, vigour index

INTRODUCTION

Citrus macroptera, also known as Satkara or Hatkora, is a fruit tree in the Rutaceae family. It is a semi-wild species of citrus that is native to Malaysia and is very widespread in India, especially in North East India, particularly in Mizoram and Manipur, where it is most frequently used as a flavouring and aromatic agent. The tree, which has thorns and may grow to a height of 5 m, is known locally as Heiribob. The rind is peeled and added fresh or dried for later use by the people of Manipur (Meitei *et al*., 2012). This tree has many long spines covering its stem, branches, and twigs. Satkara/ Hatkora are mostly used to treat infants' abdomen aches, hypertension, illness, fever, and diarrhoea (Grover *et al*., 2002). According to Rana and Blazquez (2012), the primary chemicals found in the essential oils extracted using hydro distillation from fresh *Citrus macroptera* peels include limonene, beta-caryophyllene, and geranial.Sat Kara is a medicinal plant with a wide range of pharmacological properties. Numerous parts of this plant, particularly the fruit, have a vast array of traditional medicinal uses for treating various illnesses. Many of the plant's active phytochemical components, including limonene, beta-caryophyllene, beta-pinene, geranial edulinine, ribalinine, isoplatydesmine, and others, have been identified thus far. Numerous investigations have shown the pharmacological potential of *C. macroptera's* fruits, leaves, and stems as hepatoprotective, hypoglycemic, thrombolytic, antioxidant, cytotoxic, antibacterial, and anxiolytic agents (Aktar and Foyzun, 2017).

Canellas and Olivares (2014) showed that humic acid consistently exhibits positive impacts on plant biomass and is widely acknowledged as a plant growth promoter, especially by changes in root architecture and growth dynamics, resulting in increased root size, branching, and a higher density of root hair with a larger surface area. In general, humic acid stimulation of root growth is more noticeable than stimulation of shoot growth. Devi *et al*. (2022) reported that among the different biofertilizer treatments application of *Azotobacter+ Trichoderma viride* recorded the maximum plant height, leaf area/plant, seedling vigour index-I, seedling vigour index-II, fresh weight of shoot & root, and dry weight of shoot & root in Kachai Lemon (*Citrus jambhiri* Lush.). In Manipur, it is cultivated for its fruit peel as spice and its fruit price range from Rs.20-50/piece.

Citrus macroptera is generally propagated by seed since due to lacking of vegetative propagation of this important citrus species. There is no report regarding beneficial effect of biofertilizers and plant growth stimulators on vegetative growth parameters in *Citrus macroptera* grown under soil media conditions. To find out the suitable bio-fertilizer and to know the effect of growth stimulant like humicacid an experiment was therefore taken up.

MATERIALS AND METHODS

The experiment was conducted in the year 2022–2023 at the Department of Horticulture, College of Agriculture, Central Agricultural University, Imphal, Manipur. The matured *Satkara* fruits (*Citrus macroptera)* were collected from Machi village in Tengnoupal district of Manipur. *Satkara* fruits were collected on 21st March, 2022 and seeds were extracted on the same date. After extraction, seeds were dried in laboratory or shade condition for 2 days. The seeds were treated with different treatment combination on 24th March, 2022. 10 seeds for each replication was taken and 30 seeds were treated under each treatment. The biofertilizers was purchased online from a bio-tech company. The biofertilizers were powder form of *Pseudomonas fluorescens (gm/kg of seed), liquid form of Azotobacter,* VAM, *Trichoderma viride* and Humic acid (ml/kg of seed). The seeds were sown in hydroponics on 24th March, 2022. Seedlings were raised in hydroponic condition for early germination and it was done by putting the seeds in the plastic tray and blotting paper are kept in between the thin bamboo stick. There were9 treatments: T_1- *Pseudomonas fluorescens* (5g/kg), T_2- *Azotobacter* (5ml/kg), T_3- VAM(5ml/kg), T_4- *Trichoderma viride* (5ml/kg), T_5- *Pseudomonas fluorescens* (2.5g/kg) + *Trichoderma viride* (2.5ml/kg), T_6- *Azotobacter* (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg), T_7- VAM (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg), T_8- Humic acid (Plant growth stimulator) 5ml/kg and T_9- water soaking for about 12 hours (control). Seeds were treated with different biofertilizers for about two minutes. A total of 30 seeds were utilised for each treatment. The experiment was set up using a Completely Randomised Design (CRD), with nine (9) treatments replicated three times.

All of the experimental seeds were routinely maintained and given the right amount of water (proper filling water in hydroponics condition so that water are in contact with blotting paper) during the period of the research. The sprouted seedlings were transplanted from hydroponics condition to soil condition (poly bags) about three months from date of sowing.

The vegetative growth parameters, *viz.*,plant height (cm), number of leaves/plant, leaf area/plant (cm^2), root length (cm), root diameter (mm), number of fibrous root/plant, shoot: root ratio, fresh weight of shoot and root (g), dry weight of shoot and root (g), vigour index 1, vigour index 2, survival % under soil media, volume of the root (cm^3) and chlorophyll content (mg/g) were recorded 2 months after transplanting of seedlings in soil media (poly bags).

Plant height and root length were measured by using measuring scale**.** Leaf area was calculated by using leaf area meter after 2 months of transplanting to the polybags of soil media and it was expressed in square centimetre (cm^2).It is measured with the help of digital vernier calliper. Number of fibrous root was recorded by counting number of fibrous root/plant.

Shoot: root ratio : It is recorded by using the following formulae

$$\text{Shoot root ratio} = \frac{\text{Dry weight of shoot}}{\text{Dry weight of root}}$$

Survival % under soil : It is recorded by using the following formulae

$$\text{Survival \%} = \frac{\text{No.of seedlings survived}}{\text{Total no.of seedlings planted}} \times 100$$

Volume of the root: It was measuredafter 2 months of transplanting in to soil media the saplings were uprooted with care and the volume of the roots were determined by the water displacement method. The uprooted roots after washing were dipped in a measuring cylinder containing water and the readings were recorded before and after the placement of roots. The slight increase in water level was noted as root volume for the specific treatment and expressed in cubic centimetre (cm^3).

Measurement of Chlorophyll a and b: It is recorded by using the following formulae

$$\text{Chlorophyll a} = \frac{[12.7(A663) - 2.69(A645)] \times V}{1000 \times W}$$

$$\text{Chlorophyll b} = \frac{[22.9(A645) - 4.68(A663)] \times V}{1000 \times W}$$

$$\text{Total Chlorophyll content (mg/g)} = \frac{[20.2(A645) + 8.02(A663)] \times V}{1000 \times W}$$

Where ; V = final volume of chlorophyll extract in 80% acetone

W = fresh weight of tissue extracted

Vigour index: 1 (VI-1) was calculated using the following formula given by Abdul Baki and Anderson (1973).

VI-1 = Seedling length x Germination percentage

Vigour index: 2 (VI-2) was calculated as per the formula given by Bewley and Black (1982).

VI-2 = Seedling dry weight x Germination percentage

RESULTS AND DISCUSSIONS

Germination percentage

Germination percentage 2 months after sowing indicated that (Table 1) the treatment T_8-Humic acid having performed significantly more effectively than all the other treatments, produced the greatest seed germination percentage (96.67%). T_1- *Pseudomonas fluorescens* and T_3- VAM followed, with a mean of 76.67%, in that order. T_4- *Trichoderma viride*, T_5- *Pseudomonas fluorescens*+ *Trichoderma viride* and T_7- VAM + *Trichoderma viride* all showed similar mean values of 56.67%. Besides, T_9- Water soaking (control) had the lowest seed germination rate (50%), which was extremely lower than the results from the other treatments. The results of humic acid treated *Citrus macroptera* seeds improved vegetative growth might be due to it provides the plant with basic organic nutrients and major and minor mineral elements in addition to its high ability to retain water and high oxygen content, which aids in seed germination through its role as a catalyst, allows the seed to absorb nutrients. Further, it might be due to the respiration of seed tissue cells helps the embryo quickly transition from the non-self-feeding stage to the self-feeding stage within the seed. Besides, it promotes plant growth by boosting hormones like auxin and cytokinin that help with photosynthesis, stress resistance, and absorption of nutrients. Similar finding is also reported by Hussein *et al.* (2020) and Ampong *et al.* (2022).

Plant height (cm)

The information on the impact of biofertilizers and plant growth stimulator on seedling height of *Citrus macroptera* were significantly varied between the treatments (Table 1). The treatment T_8- Humic acid (11.63 cm) have highest maximum plant height, then T_5- *Pseudomonas fluorescens*+ *Trichoderma viride* (8.57 cm). Whereas, the lowest plant height (6.0 cm) was present in T_9- water soaking (control).The results from this study indicated that foliar application of humic acid considerably affected plant height, with the maximum value for this parameter obtained under 2% humic acid foliar spray and the lowest value for this parameter recorded under control conditions (Sani, 2014).

Number of leaves/plant

In Table 1, the statistical information obtained from the current study about the number of leaves/ plant (under soil condition) is presented. The treatment T_8- Humic acid (7.33) resulted in maximum number of leaves/plant. Whereas, the lowest no. of leaves/plant was recorded in T_7- VAM + *Trichoderma viride* (3.67) as compared to the

Table 1: Effect of Biofertilizers and humic acid on vegetative growth parameters of *Citrus macroptera* seedlings

Treatment	Details	Plant height (cm)	Number of leaves/ plant	Leaf area/ plant (cm^2)	Root length (cm)	Root diameter (mm)	Number of fibrous root	Shoot: root ratio	Seed germination (%)
T_1	*Pseudomonas fluorescens* (5g/kg)	7.40	4.33	3.53	11.00	1.14	8.67 (0.93)	3.12	76.67 (61.22)
T_2	*Azotobacter*(5ml/kg)	7.13	5.00	2.87	12.03	1.18	15.00 (1.18)	2.45	60.00 (51.15)
T_3	*VAM* (5ml/kg)	8.03	5.67	3.40	12.00	1.26	12.67 (1.04)	2.85	76.67 (61.22)
T_4	*Trichoderma viride* (5ml/kg)	6.90	5.33	2.70	10.57	1.21	15.00 (1.17)	1.78	56.67 (48.93)
T_5	*Pseudomonas fluorescens* (2.5g/kg) + *Trichoderma viride* (2.5ml/kg)	8.57	5.33	3.97	13.83	1.25	19.00 (1.28)	2.27	56.67 (48.93)
T_6	*Azotobacter* (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg)	7.67	5.00	3.93	12.70	1.22	10.67 (0.96)	2.40	60.00 (50.85)
T_7	*VAM* (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg)	6.47	3.67	3.23	10.27	1.19	12.00 (1.06)	2.21	56.67 (48.85)
T_8	Humic acid (Plant growth stimulator) 5ml/kg	11.63	7.33	5.63	14.37	1.65	20.00 (1.30)	3.98	96.67 (83.86)
T_9	Water soaking (control)	6.00	4.00	2.67	9.40	1.08	6.00 (0.74)	2.14	50.00 (45.00)
	S.Em±	**0.63**	**0.51**	**0.45**	**1.01**	**0.09**	**0.10**	**0.36**	**4.28**
	CD @ 5%	**1.87**	**1.51**	**1.34**	**3.01**	**0.28**	**0.31**	**1.08**	**12.71**

*Fig in the parenthesis are square root transformed values

Tables 2: Effect of Biofertilizers and humic acid on fresh weight of shoot &root, dry weight of shoot&root, VI-1, VI-2 and survival %of *Citrus macroptera* seedlings under soil media condition

Treatment	Details	Fresh shoot weight (g)	Fresh Root weight (g)	Dry shoot weight (g)	Dry root weight (g)	Vigour index-1	Vigour index-2	Survival % under soil
T_1	*Pseudomonas fluorescens* (5g/kg)	0.25	0.08	0.061	0.023	566.00	4.70	93.33 (9.69)
T_2	*Azotobacter* (5ml/kg)	0.26	0.13	0.066	0.028	422.67	4.23	91.67 (9.60)
T_3	*VAM* (5ml/kg)	0.29	0.14	0.078	0.033	610.00	5.86	81.11 (9.02)
T_4	*Trichoderma viride* (5ml/kg)	0.25	0.17	0.067	0.038	391.00	3.82	95.24 (9.78)
T_5	*Pseudomonas fluorescens*(2.5g/kg) + *Trichoderma viride* (2.5ml/kg)	0.37	0.18	0.096	0.042	502.67	5.48	86.67 (9.34)
T_6	*Azotobacter* (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg)	0.28	0.11	0.070	0.029	465.33	4.23	82.14 (9.09)
T_7	*VAM* (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg)	0.22	0.10	0.054	0.028	339.00	2.66	93.33 (9.69)
T_8	Humic acid (Plant growth stimulator) 5ml/kg	0.89	0.20	0.234	0.059	1123.33	22.76	96.29 (9.84)
T_9	water soaking (control)	0.20	0.09	0.048	0.022	325.00	2.68	80.55 (8.99)
	S.Em±	**0.04**	**0.02**	**0.012**	**0.004**	**64.71**	**1.19**	**0.20**
	CD @ 5%	**0.13**	**0.07**	**0.034**	**0.013**	**192.26**	**3.54**	**0.60**

*Fig in the parenthesis are square root transformed values

Tables 3: Effect of Biofertilizers and humic acid on volume of the root, chlorophyll-a, b, total chlorophyll of *Citrus macroptera* seedlings

Treatment	Details	Volume of the root (cm^3)	Chlorophyll-a (mg/g)	Chlorophyll-b (mg/g)	Total Chlorophyll (mg/g)
T_1	*Pseudomonas fluorescens* (5g/kg)	0.20	0.89	0.25	1.13
T_2	*Azotobacter* (5ml/kg)	0.20	0.88	0.25	1.13
T_3	*VAM* (5ml/kg)	0.33	1.16	0.52	1.69
T_4	*Trichoderma viride* (5ml/kg)	0.22	0.99	0.32	1.31
T_5	*Pseudomonas fluorescens* (2.5g/kg) + *Trichoderma viride* (2.5ml/kg)	0.23	1.06	0.23	1.29
T_6	*Azotobacter* (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg)	0.13	1.08	0.45	1.52
T_7	*VAM* (2.5ml/kg) + *Trichoderma viride* (2.5ml/kg)	0.17	0.93	0.29	1.22
T_8	Humic acid (Plant growth stimulator) 5ml/kg	0.30	1.36	0.69	2.05
T_8	Water soaking (control)	0.10	0.84	0.20	1.04
	S.Em±	**0.006**	**0.061**	**0.027**	**0.012**
	CD @ 5%	**0.017**	**0.182**	**0.081**	**0.036**

water soaking (control). According to Mohammadipour *et al.* (2012), the best treatment for humic acid at 2000 mgL^{-1}, which resulted in 46.02 leaves per plant, whereas the least successful treatment was the control, which resulted in 30.67 leaves per plant in marigold. Further, Türkmen *et al*. (2004) reported that plant number of leaves was greatly impacted by humicacid treatments and tomato seedlings had the more leaves when HA_3 was applied.

Leaf area/plant (cm^2)

In the Table 1 clearly shows that the treatment T_8- Humic acid produced the largest leaf area per plant (5.63 cm^2), followed by T_5- *Pseudomonas fluorescens+ Trichoderma viride* (3.97 cm^2). In contrast, T_9- water soaking (control) produced the lowest leaf area per plant (2.67 cm^2). Motaghi and Nejad (2014) showed that application of 100 ppm humic acid during the vegetative growth stage resulted in the highest leaf area index compared to other treatments, whereas the control treatment had the lowest leaf area index.

Root length (cm)

In the Table 1, the maximum root length was obtained by Treatment T_8- Humic acid (14.37 cm), while the lowest root length (9.40 cm) across all the treatments was recorded by treatment T_9, water soaking (control). There was report about role of Humic acid in promoting seedlings' root growth in maize (*Zea mays*) (Canellas *et al*., 2002).

Root diameter (mm)

The data presented in Table 1 clearly show that the root diameter reacts significantly to the various treatments. The maximum root diameter (mm) was recorded in T_8- Humic acid (1.65 mm), followed by T_3- VAM (1.26 mm) and T_5- *Pseudomonas fluorescens+ Trichoderma viride* (1.25 mm). However, the treatment T_9- water soaking (control) was recoded lowest values (1.08 mm). Humic acid-Nitrogen treatment significantly increased root diameter and root surface area of sweet potato (Chen *et al.*, 2017).

Number of fibrous root/plant

The statistical analysis of Table 1, indicated that the various biofertilizers and their combinations had a significant impact on the number of fibrous roots that were observed under soil media condition. The maximum number of fibrous root (20) was obtained in T_8- Humic acid, followed by T_5- *Pseudomonas fluorescens+ Trichoderma viride* (19). The minimum number of fibrous root (6) was recorded in T_9 -water soaking (control).The effect of Humic acid treatment markedly increased the number of fibrous roots in tomato plants as compared to control (Sun *et al*., 2022).

Shoot: root ratio

Among the different treatment, T_8- Humic acid have the maximum shoot: root ratio (3.98), followed by T_1- *Pseudomonas fluorescens* (3.12) and T_3- VAM (2.85), whereas the T_4- *Trichoderma viride* gave the lowest shoot: root ratio (1.78). Meganid *et al.* (2015) reported that the shoot/root ratio increased with the addition of humic acid at 30 DAP in common bean plant.

Fresh weight of shoot and root (g)

The maximum fresh weight of shoot (0.89 g) and fresh root weight (0.2 g) was observed under soil in treatment T_8- Humic acid. However, the lowest fresh weight of shoot and root was recorded in T_9- water soaking (control) has mean values (0.20 g) and T_1- *Pseudomonas fluorescens* (0.08 g), respectively. The application of HA_2 led to the highest shoot and root fresh weights in tomato seedlings was observed by Türkmen*et al*. (2004). According to Meganid *et al.* (2015) results showed that at 15, 30, and 45 days after planting, humic acid application greatly raised the fresh and dry weights of the shoots and roots of common bean plant as compare to the control.

Dry weight of shoot and root (g)

The T_8- Humic acid had the maximum dry weight of shoot (0.234 g) and dry root weight (0.059 g), while the lowest dry weight of the shoot and root (0.048 g) & (0.022 g) was observed in T_9- water soaking (control). Türkmen *et al*. (2004) also reported that tomato (*Lycopersicon esculentum* L.) seedlings under saline soil conditions shoot and root dry weights were higher at HA_2 application than at other levels.

Vigour index-1 (VI-1) and Vigour index-2 (VI- 2)

The various treatments have a significant influence on the VI-1 and VI-2. Among the different treatment, the highest VI-1 (1123.33) and VI-2 (22.76) was observed under the T_8- Humic acid, while lowest mean VI-1 and VI-2 was found under the T_9- control (water soaking) and T_7- VAM + *Trichoderma viride*, (325) & (2.66), respectively. Ali *et al*. (2020) reported that Humic acid exhibited most impacts on the seedling vigour index in sorghum plant. According to Weerasekara *et al.* (2021) priming soybean seeds treated with humic acid for 3,5 and 7 hours which enhances the germination with the increase of time and increases the seedling vigour index as compared to control.

Seedlings survival (%)

The maximum survival % under soil condition (96.29 %) was observed in the treatment T_8- Humic acid followed by T_4- *Trichoderma viride* (95.24). The treatment T_9- water soaking (control) obtained the lowest mean values (80.55 %). According to Justi *et al.* (2019), plants treated with humic acid may have a higher chance of surviving of coffee seedlings in the field. Nasratullah (2020) observed that the application of humic had the largest number of survival seedlings (98.67%) of Rangpur Lime as compared to the control.

Volume of the root (cm³)

The maximum root volume among the various treatments was reported in T_3- VAM (0.33 cm^3), which was noticeably greater compared to all other treatments. T_8- Humic acid came second, with a root volume of (0.30 cm^3) and it was statistically at par with T_3. In T_9- water soaking (control), the lowest root volume (0.10 cm^3) was recorded. The growth of a prolific root system during treatment may be the cause of the larger root volume. VAM expand their hyphae into the host root system and assist in the expansion of the plant's root system, which improves the plant's ability to absorb nutrients & water, and also helps in root growth and stimulates the growth of the whole plant. Maximum root volume (10.34 cm^3) under the treatment of *Azospirillum*, PSB, *Pseudomonas fluorescens*, and VAM was recorded by Fayaz *et al.* (2020) at 120 days in pummelo seedlings.

Chlorophyll content (mg/g)

It is clearly shown, that the treatment T_8- Humic acid had the highest mean value of the treatments, producing the highest amount of chlorophyll-a (1.36 mg/g), chlorophyll-b (0.69 mg/g), and total chlorophyll (2.05 mg/g), followed by T_3- VAM, which recorded chlorophyll-a (1.16 mg/g), chlorophyll-b (0.52 mg/g) and total chlorophyll (1.69 mg/g). while, treatment T_9- water soaking (control) had the lowest mean values for chlorophyll-a (0.84 mg/g), chlorophyll-b (0.20 mg/g), and total chlorophyll (1.04 mg/g) in comparison to other treatments. Meganid *et al.* (2015) showed that application of humic acid at 15, 30, and 45

DAP respectively in common bean plants had considerably higher chlorophyll levels than control plants. According Karakurt *et al*. (2009) application of humic acid had a significant impact on the total chlorophyll concentration, with chlorophyll b having the most significant impact in pepper plant. Further, Ali *et al.* (2020) also revealed that seeds treated with 6 g of humic acid per kg^{-1} of soil resulted in maximum chlorophyll-b and total chlorophyll in sorghum seedlings at a salinity level of 200 mM NaCl.

CONCLUSION

From the present investigation, it was found that the different biofertilizers singly and their combination and humic acid have positive effects on growth parameters of *Citrus macroptera* seedlings. Among the various treatments, it was observed that humic acid @ 5 ml/kg executed the best result with respect to plant height, number of leaves/plant, leaf area/plant, root length, number of fibrous root, root diameters, shoot: root ratio, fresh weight of shoot & root, dry weight of shoot & root, vigour index-1 & 2, survival % under soil condition, chlorophyll-a, b & total chlorophyll, so that it can be used for obtaining a higher number of seedlings of *Citrus macroptera.*

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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