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Compost Tea Induced Callus Proliferation and Defense Response in Rice (*Oryza sativa* L.) Callus Cells

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A B S T R A C T

Callus growth and vitality is one of the important prerequisites for further success of *in vitro* studies. In vitro calli systems can also act as a model system in understanding defense responses at the cellular level. This study investigated the efficiency of Compost tea (CT) in inducing defense response and as an organic supplement *in vitro* callus growth in rice. Compost tea is an organic liquid product derived from quality compost, carrying useful microorganism and molecules capable of protecting and stimulating the growth of the plants. Morphological and Biochemical responses of rice calli inoculated onto Murashige and skoog medium supplemented with CT in the presence or absence of 2,4-D showed significantly high callus proliferation, fresh weight of calli, Peroxidase (POX) and Superoxide dismutase (SOD) activity after 15 days of inoculation as compared to controls. Interestingly MS media supplemented only on MS media supplemented with CT and without 2, 4 D showed rooting/root like growth from calli. The observed callus proliferation, and root like growth suggesting presence of auxin like molecules in compost tea. The study shows that compost tea can induce a cellular defense response. Secondly under the light of the findings, compost tea can be used as an organic bio stimulant for efficient callus growth and complementing commercial chemical hormones in rice.

Introduction

Rice (*Oryza sativa* L.) is the most important staple food crop which is central to the lives of billions of people around the world. Rice has shaped the culture, diets and economies of millions of people. Compost tea (CT) is a watery suspension made by steeping compost in water and fermented under aerated or non aerated conditions. CT is an enriched microbial liquid suspension and contains growth promoting biomolecules produced by microbes (Anil *et al.*, 2017a; Weyens *et al.*, 2009).

Microbial synthesis of gibberellins and gibberellin like molecules has been reported in fungi, bacteria and actinomycetes (Katznelson and Cole., 1965). Production of IAA has been reported from many bacteria. It is even assumed that over 80 % of the bacteria isolated from the rhizosphere are capable of IAA synthesis (Khalid *et al.*, 2006; Spaepen and Vanderleyden, 2011). Cytokinins (CK),

auxins, gibberellins (GAs) and brassinosteroids (BRs) in three batches of vermicompost leachate (VCL) were quantified Aremu *et al.,* (2015). Hormone-like molecules, including gibberellins, indoleacetic acid and cytokinins that were identified in highly bioactive compost tea (Bernal-Vicente *et al.,* 2008; Pant *et al.,* 2012; Anil *et al.,* 2017).

Compost tea is also known to suppress disease in several crops. Compost watery extracts (CWEs) sprayed on pepper and cucumber plants were showed increased activity of defense enzymes *Viz*., POX, β-1,3-glucanase, chitinase under pathogen-inoculated conditions (Sang and Kim, 2011). Anil and coworkers have demonstrated the induction of defense enzymes superoxide dismutase and peroxidase in potato, groundnut, marigold and rice crops (Unpublished data). The induction of a systemic resistance in plants due to exposure to compost tea needs to be evaluated at the cellular level as well.

In recent years *in vitro* techniques have been extensively used not only for *in vitro* screening in plants against abiotic/biotic stresses but also creating *in vitro* models for studying and observing morphological, physiological and biochemical changes of both unorganized cellular (such as suspension cultures and callus cultures) and organized tissue (such as axillary shoot, shoot tip, mature embryo, and whole plant) levels. Activities of defensive enzymes peroxidase (POX), superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO) and esterase (EST) and their isozymes in pear calli were studied to reveal their role in the defensive response to different fungal infections and to find some clues to enhance their antimicrobial properties (Zhao *et al.,* 2012).

This study was conducted to investigate the contribution of compost tea as an organic bio stimulant on callus growth in rice. It also uses the calli as a model system to demonstrate compost tea induced defense responses. To our best knowledge, this is the first report to determine the effect of compost tea as an organic bio stimulant on callus growth in rice.

Materials and Methods

Callus induction

Rice calli were induced from ARB 6, BR2655, HR12 and KRH 4 rice genotypes. Dehusked and surface sterilized rice seeds were cultured on MS solid medium (Murashige and Skoog, 1962), containing 2 mgl^{-1} 2, 4-D and allowed to germinate and dedifferentiate. Calli that initiated at the radicle-endosperm juncture were excised and allowed to further proliferate in medium containing 1 mgl^{-1} 2, 4-D for two weeks (Anil *et al.,* 2007). All cultures were incubated at 25±1°C under cool white fluorescent light (about 2,000 lux) with a 16 h light/8 h dark photoperiod. The pH of the medium was adjusted to 5.6-5.8 prior to autoclaving.

Preparation of compost tea media

Two dominant approaches are being advocated in compost tea production. They are aerated and non aerated methods. Aerated compost tea (ACT) is made by keeping compost with water in the ratio of 1:10 with the supply of oxygen for one week where Non-Aerated compost tea (NCT) is prepared in the same manner without active aeration. Compost tea medium was prepared by adding ACT and NCT to MS medium at 10% concentration, and autoclaved. The experiments has done with six treatments (Table 1) and three replicates as follows,

Packed cell volume of 100 µl of callus of each of the four genotypes was inoculated on each of the above treatments in petriplates by

placing each of the callus equidistant from each other. The calli were allowed to grow for a period of 12-15 days in the tissue culture room under defused light conditions. The callus growth was evaluated visually and by the FW. The defense enzyme activity induced by compost tea at cellular level was monitored on 15 days post inoculation.

Protein extraction

Calli from different treatments were frozen in liquid nitrogen, to prevent proteolytic activity, and homogenized using a mortar and pestle. The homogenate was then suspended in extraction buffer [Phosphate buffer 0.1 M, pH 7.8, 1mM PMSF (protease inhibitor) and 0.1 percent of poly vinyl pyrrolidone (PVP)] and kept on ice for 15 min. The crude protein extracts were centrifuged at 14,000 rpm at 4°C for 30 min. The pellet was discarded and the supernatant containing the soluble proteins was used for further experiments. Protein concentration was determined by the method of Lowry (Lowry *et al.,* 1951) using BSA as standard.

Defense enzyme assays

Peroxidase enzyme activity in the protein extract was measured by the method proposed by Castillo *et al.,* (1984) with slight modification. Peroxidase activity is assayed as increase in optical density due to the oxidation of guaiacol to tetra-guaiacol. Native PAGE was performed as the method described by Davis (1964) for peroxidase isoenzyme activity by using 10 % resolving gels and 5 % stacking gel. Protein extract (25 µg) of all genotypes and treatments were loaded in gel. Electrophoresis is performed initially at 60 volts and when the protein entered the resolving gel, the voltage was increased to 120. Electrophoresis was conducted at 40C for about 3h. Later the gel was stained for peroxidase isoenzymes.

SOD activity was measured by the method described by Dhindsa *et al.,* (1981) with slight modifications. SOD activity in the supernatant was assayed by its ability to inhibit photochemical reduction of nitroblue tetrazolium. Native PAGE was performed according to the method described by Davis (1964) for superoxide dismutase isoenzyme activity by using 5 % stacking gels and 10 % resolving gels. Protein extract (25 µg) from all genotypes and treatments were loaded to gel. Electrophoresis was performed initially at 60 volts and after the protein entered the resolving gel the voltage was increased to 120.

The electrophoresis was conducted for 3 h at 40C. The gel was incubated in a staining solution containing 100 % NBT (w/v), 0.2M EDTA (w/v), 0.1M sodium phosphate buffer (pH 7.5), commercial grade TEMED and 5 % riboflavin (w/v) for 30 min until the bands appeared. The isoenzyme bands appeared as white/colourless in a dark blue background and the isoenzyme pattern was photographed.

Statistical analysis

Factorial CRD, statistical design was used to conduct the experiment. Web Agri Stat Package 2.0 (WASP) and OPSTAT online software systems were used to analyze the data.

Results and Discussion

MS plain media acts as the control to MS with 10 % NCT and MS with 10 % ACT media. MS + 2,4-D media acts as control to MS NCT+ 2,4-D and MS ACT + 2,4-D media. These compost tea media and combination of 2,4-D with CT media showed significantly high fresh weight of calli, POX and SOD activity in all 4 genotypes compared to control media at 1 % level of significance (Table 2, 3 and 4).

Morpho-physiological and growth responses of callus to compost tea

Rice calli grown on compost tea media resulted in a significant higher fresh weight after 15 days of incubation indicating callus proliferation (Table 2 and plate 1).

In genotype ARB 6, treatment MS ACT (59.67 mg) showed significantly higher fresh weight than control *i.e.,* MS (49.00 mg). In genotype BR 2665, treatment MS ACT (69.67 mg) showed significantly higher fresh weight compared to control *i.e.,* MS (46.33 mg). In HR 12 genotype, MS ACT (79.00 mg) was significantly higher in fresh weight than control *i.e.,* MS (45.67 mg). In genotype KRH 4, treatment MS ACT (66.00 mg) showed significantly higher fresh weight than control *i.e.,* MS (51.00 mg). Similarly NCT Media also resulted in higher calli proliferation than the control, but in all genotypes ACT had a better effect on callus proliferation. Overall 17 to 42 % increase in fresh weight was observed in compost tea media compared to control plain MS media. Rooting of calli was observed on MS medium with 10 % ACT and in MS medium with 10 % NCT (Plate 01).

In genotype ARB 6, treatment MS+ACT+ 2,4-D (81.67 mg) showed significantly higher fresh weight than control *i.e.,* MS+ 2,4-D (58.33 mg). In genotype BR 2655, treatment MS+ ACT+ 2,4-D (78.67 mg) showed significantly higher fresh weight than control *i.e.,* MS+ 2,4-D (49.33 mg). In genotype HR 12 treatment MS+ ACT+ 2,4-D (82.67mg) showed significantly higher fresh weight than control *i.e.,* MS+2,4D (48.00 mg).In genotype KRH 4, treatment MS+ ACT+ 2,4-D (82.33 mg) showed significantly higher fresh weight than control *i.e.,* MS+ 2,4-D (54.00 mg). Overall 28 to 41 % increase in fresh weight was observed in CT+ 2,4-D media compared to control *i.e.,* MS + 2,4-D media. Similarly NCT Media also resulted in higher calli proliferation than the control, but in all genotypes ACT had a better effect on callus proliferation (Table 2).

Effect of compost tea on the activities of POX and SOD activities in calli cells

Peroxidase activity in calli

Calli grown on MS NCT showed high activity of POX as compared to control treatment, but was on par with calli grown on MS ACT (Table 3). In ARB 6 genotype, treatment MS NCT resulted in higher POX activity (358.33 µg/mg protein) than control *i.e.,* MS (226.75 µg/mg protein). In BR 2655 genotype, treatment MS NCT resulted in higher POX activity (362.33 µg/mg protein) than control *i.e.,* MS (280.33 µg/mg protein). In HR 12 genotype, treatment MS NCT resulted in higher POX activity (598.55 µg/mg protein) than control *i.e.,* MS (496.77 µg/mg protein). In KRH 4 genotype MS NCT resulted in higher POX activity (397.84 µg/mg protein) than control *i.e.,* MS (301.67 µg/mg protein). Overall 17 to 36 % increase in activity of POX was observed in compost tea media compared to control plain MS media (Table 3 and plate 02).

Treatment MS+ NCT+ 2,4-D resulted in calli with high activity of POX as compared to controls but was on par with MS+ ACT+2,4-D. In ARB 6 genotype, treatment MS+ ACT+2,4-D resulted in higher POX activity (372.33 µg/mg protein) than control *i.e.,* MS +2,4-D (235.00 µg/mg protein). In BR 2655 genotype MS+ ACT+2,4-D resulted in higher POX activity (350.39 µg/mg protein) than control *i.e.,* MS +2,4-D (269.33µg/mg protein). In HR 12 genotype, treatment MS+ NCT+ 2,4-D resulted in higher POX activity (630.77µg/mg protein) than control *i.e.,* MS +2,4-D (534.06 µg/mg protein). In KRH 4 genotype, treatment MS+ ACT+2,4-D resulted in higher POX activity (426.12 µg/mg protein)

than control *i.e.,* MS +2,4-D (308.00 µg/mg protein). Overall 15 to 36 % increase in POX activity was observed in compost tea+2,4-D media compared to control *i.e.,* MS +2,4-D media. The compost tea induced enhancement of POX was comparable in plus 2,4D and minus 2, 4-D treatments, indicating that 2,4-D by itself was not inducing POX activity.

Superoxide dismutase (SOD) activity in calli

MS NCT showed high activity of SOD compared to controls but was comparable with MS ACT. In ARB 6 genotype, treatment MS ACT showed significantly more SOD activity (32.76 µg protein for 50 per cent inhibition) than control *i.e.,* MS (52.11 µg protein for 50 per cent inhibition). In BR 2655 genotype, treatment MS ACT showed significantly higher SOD activity (32.76 µg protein for 50 per cent inhibition) than control *i.e.,* MS (54.79 µg protein for 50 per cent inhibition). In HR 12 genotype, treatment MS NCT showed significantly higher SOD activity (32.05 µg protein for 50 per cent inhibition) than control *i.e.,* MS (49.48 µg protein for 50 per cent inhibition). In KRH 4 genotype, treatment MS ACT showed significantly more SOD activity (37.45 µg protein for 50 per cent inhibition) than control *i.e.,* MS (50.79 µg protein for 50 per cent inhibition). Overall 25 to 39 % increase in activity of SOD was observed in compost tea media as compared to control plain MS media at 1 % level of significance (Table 4 and Plate 02).

In ARB 6 genotype, treatment MS ACT+2,4-D showed significantly higher SOD activity (27.09 µg protein for 50 per cent inhibition) than control *i.e.,* MS +2,4-D (50.12 µg protein for 50 per cent inhibition). In BR 2655 genotype, treatment MS NCT+ 2,4-D showed significantly higher SOD activity (30.56 µg protein for 50 per cent inhibition) than control *i.e.,* MS +2,4-D (53.89 µg protein for 50 per cent inhibition). In HR 12 genotype, treatment MS NCT+2,4-D showed significantly higher SOD activity (26.92 µg protein for 50 per cent inhibition) than control *i.e.,* MS + 2,4-D (50.09 µg protein for 50 per cent inhibition) . In KRH 4 genotype, treatment MS ACT+ 2,4-D showed significantly higher SOD activity (29.04 µg protein for 50 per cent inhibition) than control *i.e.,* MS +2,4-D (49.01µg protein for 50 per cent inhibition) at 1 % level of significance. Overall 40 to 46 % increase in activity of SOD was observed in compost tea media compared to control MS+ 2,4-D media at 1 % level of significance. The Compost tea induced enhancement of SOD activity was consistently higher when the experiment was carried out in the presence of 2,4-D.

Table.1 Compost tea media used for callus growth

T1	**Plain MS medium (control)**
T2	MS medium with 2 mg/l 2,4-D
T3	MS medium with 10% ACT
T4	MS medium with10% NCT
T5	MS medium with 10% ACT+ 2 mg/l 2,4D
T6	MS medium with 10% NCT+2 mg/l 2,4-D

Table.2 Fresh weight of callus after 15 days growth on CT media

	Treatments	Fresh weight of calli (mg)			
		ARB 6	BR 2655	HR 12	KRH 4
T1	MS	49.00	46.33	45.67	51.00
T2	MS 2,4-D	58.33	49.33	48.00	54.00
T3	MS ACT	59.67	69.67	79.00	66.00
T4	MS NCT	55.67	49.67	51.67	58.00
T5	MS+ ACT +2,4-D	81.67	78.67	82.67	82.33
T6	MS +NCT +2,4-D	64.67	53.67	69.00	65.67
		S.Em ±	CD at 1 %	CV	
	Varieties	0.73	2.69	4.921	
	Treatments	0.90	3.29		
	T×V	1.79	6.59		

Table.3 Peroxidse enzyme activity in rice Calli after 15 days growth on compost tea media

	Treatments	Peroxidase (µg/mg protein)			
		ARB 6	BR 2655	HR 12	KRH 4
T1	MS	226.75	280.33	496.77	301.67
T2	MS 2,4D	235.00	269.33	534.06	308.00
T3	MS ACT	306.44	322.53	576.56	348.00
T4	MS NCT	358.33	362.33	598.55	397.84
T5	MS+ ACT+ 2,4D	372.33	350.39	627.87	426.12
T6	MS +NCT +2,4D	358.00	338.83	630.77	412.67
		S.Em ±	CD at 1 %	CV	
	Varieties	1.176	4.492	2.611	
	Treatments	1.441	5.502		
	T×V	2.881	11.004		

Table.4 Superoxide dismutase enzyme activity in rice Calli after 15 days growth on compost tea media

	Treatments	Superoxide dismutase (µg protein for 50 per cent inhibition)			
		ARB 6	BR 2655	HR 12	KRH 4
T1	MS	52.11	54.79	49.48	50.79
T2	MS 2,4-D	50.12	53.89	50.09	49.01
T3	MS ACT	32.76	32.76	33.88	37.45
T4	MS NCT	34.54	33.21	32.05	38.98
T5	MS+ ACT+ 2,4-D	27.09	32.76	27.08	29.04
T6	MS +NCT +2,4-D	27.99	30.56	26.92	30.05
		S.Em ±	CD at 1 %	CV	
	Varieties	0.47	1.80	5.253	
	Treatments	0.58	2.20		
	T×V	1.16	4.41		

Plate.1 Calli proliferation on Compost tea media: A, Media without supplemented 2,4-D; Medium supplemented with 2 mg/L 2,4-D

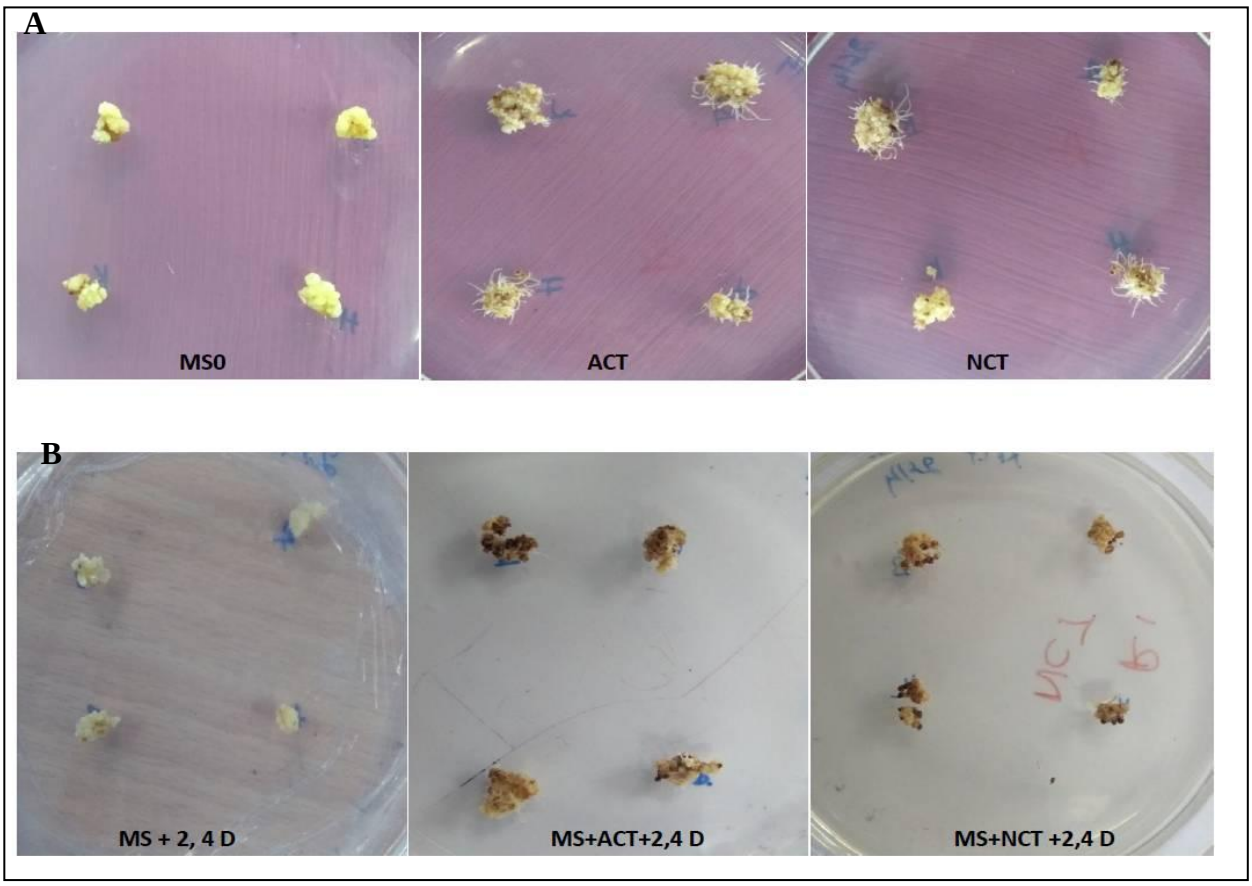
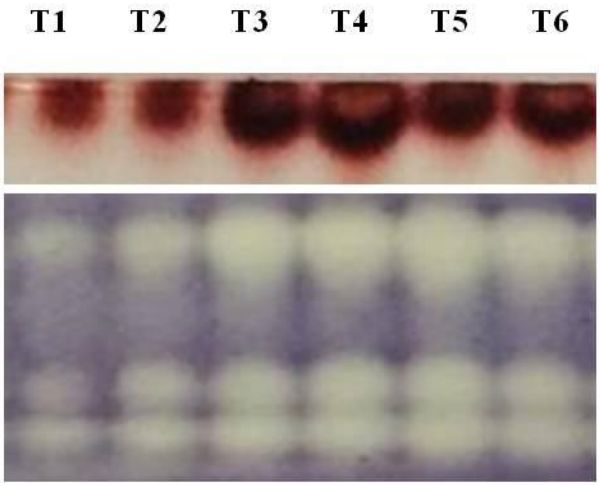


Plate.2 POX and SOD enzymes activity in rice Calli after 15 days growth on CT media: T1: MS 0, T2: MS+ 2,4-D, T3: MS+ ACT, T4 : MS+ NCT, T5: MS+ACT+2,4-D,T6: MS+NCT+2,4-D,



Compost tea has been observed to enhance plant biomass, chlorophyll content, and enhance yield in potato crop (Anil *et al.,* 2017c) and in other crops such as lettuce, soybean and sweet corn (Kim *et al.,* 2015). Compost tea thus is a plant growth promoter, and earlier studies have demonstrated the presence of IAA, GA and other phytohormone like molecules (Bernal-Vicente *et al.,* 2008; Pant *et al.,* 2012; Anil *et al.,* 2017b). Compost tea is also known to suppress plant diseases and the induction of defense proteins and secondary metabolites have been reported (Anil 2017, and Sang and Kim (2011)). In this scenario, this study uses the callus model system to test both the growth promoting properties of compost tea and its ability to induce a defense response in the plant cells.

Exogenous application of commercially produced chemical hormones (auxin and cytokinin) has been widely used to generate callus in various plant species (Ikeuchi *et al.,* 2013). Differently, in the present study, the effect or contribution of compost tea as an organic bio stimulant on callus growth and development is investigated. Calli grown on NCT and ACT media showed higher calli fresh weight and also showed root initiation and root like growth (T3 and T4) as compared to controls T1 and T2. These changes observed corroborate the detection of growth regulators (phytohormones) in compost tea (Anil *et al.,* 2017b). The induction of roots from rice calli in presence of ACT or NCT indicates that rooting hormones such as IBA may also be present in compost tea. Several reports showed the presence of growth hormones such as gibberellins, indoleacetic acid and cytokinins in compost tea (Bernal-Vicente *et al.,* 2008; Pant *et al.,* 2012; Anil *et al.,* 2017b). Similarly Beyaz and Turkay (2019) investigated morpho-physiological responses of sainfoin calli to vermicompost tea. They found that a combination of plant growth regulators (4 mg/L BAP and 0.5 mg/L NAA) with 20 % of vermicompost tea causing significant callus initiation and growth in sainfoin stem explants.

Similar to the investigation of Zhao *et al.(* 2012), present study of calli grown on compost tea media, showed higher activity of defense related molecules such as POX and SOD that indicates the ability of CT to stimulate defense priming at cellular level. Microbial metabolites in the autoclaved CT

can trigger the calli to produce defense enzymes emulating the scenario in the whole plant level were this can lead to an effective suppression of disease in crop plants.

In conclusion, the results of present study show that calli of all rice genotypes used in this study responded to compost tea media and showed significantly high callus proliferation and increased activity of defense enzymes compared to control media. Rooting observed in calli in compost tea media, hints on the presence of IBA like growth molecules in compost tea and may influence rooting in the whole plant system as well. The evaluation of the effect of compost tea on the root system of crops especially rice is warranted and will be taken up in the future. On the other hand, we assume that the protocol presented in this study could be used for other plants that are recalcitrant to manipulation *in vitro*, to obtain highest callus growth and development *in vitro*.

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