Research Article

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Biosignalling Molecules and Biomass Documentation of Casuarina Equisetifolia Inoculated with Frankia

S. Murugesan*, N. Senthilkumar, R. Sumathi, D. Suresh Babu and R. Lakshmi Devi

Division of Bioprospecting, Institute of Forest Genetics & Tree Breeding, Coimbatore, Tamilnadu, India.

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Abstract: Hopanoids are intuitive secondary biosignalling molecules of which are nitrogen fixing actinomycete symbionts inducing root nodules in a diverse group of about 200 species. *Casuarina equisetifolia* is an economically important tree nodulated by nitrogen fixing bacteria, *Frankia.* Some of the bioactive secondary molecules were found to be more important for establishing the root-associated growth of *Frankia* due to the presence of a mixture of mono- and sesqui-terpenoids, and fatty acid derivatives. Over 56 compounds were detected from different day culture fractions of *Frankia* using GC-MS-MS, and a total of 19, 23 and 14 components were characterized and identified in 15, 25 & 30th day cultures respectively. Some of the derivatives retained at 15th day were reported to produce vesicles in roots of *C. equisetifolia*. The analysis indicated variations in the presence of biotransformed signaling molecules, especially the hexa decanoic acid, phthalic acid and their derivatives at different stages of its growth period. The compounds, such as isoterpinolene, 2, 4, Phenol-bis(1, 1-dimethyl ethyl) and 1-Dotriocantanol are produced in later stages of *Frankia* growth period tend to restrict the oxygen disturbances in the nitrogenous activity and thereby enhancing nodulation and nitrogen fixation in *C. equisetifolia.* The ureides, allantoin and allantoic acid, represents major fractions of the soluble nitrogen pool of nodulated *C.equisetifolia* throughout vegetative and reproductive growth. Allantoin content was profoundly high for the *C.equisetifolia* seedlings treated with 25th day *Frankia* culture as compared to 15th, 30th day cultures. Biosignalling compounds identified from *Frankia* culture were found to increase biomass of casuarina, especially the plant height, nodule number and nodule weight. In the present study, it was found that the *Frankia* has produced a complex of bioactive compounds, like hoponoids and terpenoids at different stages during its growth period. Their role in plant-pathogen and plant-insect interactions is being studied to determine their potential in pest control.

Keywords: *Casuarina equisetifolia*, *Frankia*, Hopanoids, GCMS and Biomass.

INTRODUCTION:

Endophytes (fungal and bacterial organisms) have attracted attention over the past few years due to their presence as a source of secondary metabolites in the regulation of plant communities, particularly casurinas in terms of nodulation, biomass, growth etc. Their role in plant-pathogen-insect interactions is much more focused because of the effectiveness, however, little is known about the physiology and regulation processes of the plant-endophyte interaction especially during sporulation. The quality of allelochemicals produced by a plant is determined in part by the availability of limiting resources such as nutrients. Some plants rely on constitutive defenses, while others mobilized defenses only when

induced by tissue damage. Plants cannot move to escape environmental challenges, but in turn, have evolved sophisticated mechanisms to perceive the extraneous influence and translate into an adaptive response. The long list of secondary metabolites includes pigments such as carotenoids and glutamine, hopanoids, lipids and a diversity of other compounds of which many of them are proved to be effective in establishment of nodulation in casurinas, and yet to be identified many more are Gram-positive, aerobic, nitrogen-fixing bacteria contains lipid components called hopanoids mediate mutualistic associations between plants with the formation of a new root organ, prenodule and fixes atmospheric nitrogen. Hopanoids, which are amphiphilic, pentacylic triterpenoid lipids, condense membrane lipids for stabilizing the membranes in a similar way as sterols do in higher organisms [1]. There are many structural variants of hopanoids like polyol- and glyco-derivatives that can be found within various bacteria; the specific functions of each hopanoid derivative have not been found [2]. In specifically, membranes containing hopanoids surround vesicles called diazovesicles that contain nitrogenase, an oxygen-sensitive enzyme. While hopanoids thicken and stabilize the walls which help to keep oxygen away from the nitrogenase, some suggest that the hopanoids themselves play a more specific and molecular role in the oxygen protection mechanism of nitrogenase [3]. For example, with the ability to fix nitrogen, the casuarina species has potential use in agro-forestry, and casuarinas pulpwood alone transmutes into the annual production of 200 Crores revenue. *C. equisettifolia* become impeccable as it is resistant to the force of tidal wave occurred in the recent past. C. *junghuhniana* has given much attention because of its fast growing nature, free from diseases and high economic value too. *Frankia* strains covers the production of many secondary products, and play an important role in the regulation of plant communities in terms of growth and resistant to their herbivores. To date no literatures are available on the bioconversion of secondary plant products by bacterial spores of strains, and the efficiency needs to be tested for nodulation establishment in casuarinas.

MATERIALS AND METHODS:

***Frankia* culture establishment:** *Frankia* strains were isolated based on method described by Stone Edward *et al*. [4] and Shipton and Burgraaf [5] with some modifications. The isolated strains were identified microscopically by its morphological structures such as hyphae, vesicles, sporangia and spores. *Frankia* strain was grown in static Benzyl Amino Purine (BAP) minimal medium supplemented with Sodium pyruvate as carbon source [6] and maintained at 28ºC for 4 weeks. The homogenized cell suspension different day's culture (15^{th}, 25^{th} and 30^{th}) of *Frankia* were lyophilized and used for experimental analysis.

Plant culture and experimental conditions: *Casuarina equisetifolia* and *Casuarina junghuhniana* seedlings (one month old) in poly bags were used for experiment. Different day's pure *Frankia* cultures (15^{th}, 25^{th} and 30^{th} days) were homogenized and 5 ml of this suspensions were applied to individual seedling. Similarly individual compounds isolated and identified were also used for the same experiment with due replications. All poly bags were flushed daily with 1/4 strength nitrogen free Hoagland solution. The treated roots were collected after 30 days and subjected to biochemical investigation.

Metabolite quantification: The lyophilized sample of the different day's *Frankia* culture (15^{th}, 25^{th} and 30^{th}) was taken for the bio-chemical analysis. The root sample of the *C. equisetifolia* and *C.junghuhniana* seedlings treated with Frankia different day culture and identified individual biosignalling compounds were collected after 30 days and subjected to biochemical investigation.

Soluble protein was measured according to Lowry *et al*., [7], carbohydrate was estimated using the method of Sadasivam and Manickam [8], phenol, phenolics and flavonoids were quantified by the method described by Mc Donald *et al*., [9], lipids using the method described by Nichols [10] and Indole acetic acid was quantified according to Knegt and Bruinsma [11].

Allantoin and Allantoic acid: Allantoin and allantoic acid predominated in the soluble nitrogen fraction of nodules were quantified by the method described by Richard and Larry [12].

Enzymatic Assays: *Glutamate dehydrogenase* (GDH) and *Glutamate synthase*, the major assimilatory enzymes for the production of ammonia through nitrogen fixation in legume nodules were quantified using the methods of Delma Doherty [13] and Van de Casteele et al., [14] respectively.

Preparation of extracts for chromatographic analysis: 5ml of the homogenized suspension of the different day (15^{th}, 25^{th} and 30^{th} days) *Frankia* cultures with equal volume of methanol and 2g each of control and treated roots of *C. equisetifolia* and *C. junghuhniana* seedlings soaked in methanol were incubated for 24 hrs at room temperature. Centrifuged at 5000 rpm and collected the supernatant. Solvent was removed in *vacuo* at 40 ^{o}C on a rotary evaporator yielding crude methanol extract. The extract was dissolved in hexane and filtered through solid phase extraction column and stored at-20^{o}C for further analysis.

GC-MS-MS analysis: GC-MS-MS analysis was performed on a Varian 4000 MS coupled with a Varian 3800 GC, equipped with a cross linked 5% Phenyl 95% dimethyl polysiloxane VF-5MS capillary column (30 m x 0.25 mm i.d, film thickness, 250nm) and operating under the conditions as mentioned below: The oven temperature was programmed as 60°C (10 min), 60°C - 220°C (4°C/min), 220°C (10 min) and 220°C -240°C (1°C/min). Injector and detector temperatures were maintained at 60°C and 240°C respectively. The amount of the sample injected was 1.0 µl in the splitless mode. Helium was used as carrier gas with a flow rate of 1ml/min.

Identification of phytocompounds: Interpretation on mass-spectrum of GC-MS-MS was conducted using the database of National Institute of Standard and Technology (NIST) having more 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, molecular formula, retention time and retention indices of the components of the test materials were ascertained.

RESULTS AND DISCUSSION:

Metabolic profiles of Frankia culture: The process of nitrogen fixation is highly dependent on an adequate energy supply which is due to shuttling of dicarboxyclic acids from the host cells cytoplasm to *Frankia* and the O_2 pressure because variable *Frankia* lipidic cell envelope, expression of hemoglobin, like protein, variable respiration rates and regulation of O_2 diffusion through nodule intercellular spaces [15]. The details of biochemical budgeting in different days of *Frankia* culture and its influence on biomass in casuarina are estimated (Table 1); where the availability of carbohydrates, newly synthesized carbohydrates due to nitrogenase activity is higher in 25^{th} day culture and in roots/nodules of casuarina play a vital role in symbiosis [16]. Carbohydrates in the form of sucrose received from the nodules get reduced by the enzyme and utilized as substrate for dicarboxyclic acids synthesis and assimilation of fixed ammonia into amides and/or ureides [17].

There is no much variation in phenol and phenolic profiles as they are supposed to enhance nitrate reductase and glutamate dehydrogenase activities [18], the enzymes which trigger the assimilation of ammonia into amino acids in the nodules [19]. High quantity of flavanoids was found in 25th day culture play a crucial role in nodulation once the bacteria enter the plant and nodulation of auxin transport during the initiation of nodule primordial as well as the chemical mediate signal exchange between the symbiotic partners and isoflavanoid compounds exuded through the plant roots [20]. In alfalfa the Flavanoid, luteolin limits root nodulation, N_2 fixation, and seedling growth [21]. Flavonoids not only required to signal symbiotic bacteria [22], also play an important role in direct root nodule organogenesis. Different flavonoid classes play distinct important role in plant in establishing root nodule [22]. Plants that grow without nitrogen fertilization developed large numbers of nodules and showed strong transporter expression in the nodules and the roots. It corroborates our findings that high quantity of allantoin was found in 25th day *Frankia* culture, and its expression level was reduced in ammonium nitrate and fed allantoin at the root zone, due to the supply of dicarboxylates to the bacteroid to fuel nitrogenase for the supply of ammonium to the plant [23]. Amino acids increases from 15th to 25th day and decreased in 30th day culture because of glutamate dehydrogenase which is active from 15th to 30th day in the oxidation of glutamate (Table 1), but not in the reductive amination of 2-oxoglutarate. The enzyme glutamate dehydrogenase is derepressed in carbon limited cells and in such cells the function of glutamate dehydrogenase appears to be the oxidation of glutamate, thus ensuring sufficient carbon skeletons for effective functioning of the tricarboxylic acid cycle. This catabolic role for glutamate dehydrogenase implies an important regulatory function in carbon and nitrogen metabolism [24]. The multiple roles of glutamate in nitrogen balance make it a gateway between free ammonia and the amino groups of most amino acids. Glutamate synthase allows for the conservation of bicarbonate ion since the incorporation of ammonia into urea requires bicarbonate. The ammonia will ionizes to ammonium ion (NH4+) which is excreted. The net effect is a reduction in the concentration of hydrogen ion, [H+], and thus an increase in the pH [25].

GC/MS/MS budgeting of Frankia culture: The chemical composition of the *Frankia* cultures was found to be a mixture of mono- and sesqui-terpenoids, and fatty acids (Table 2). Over 56 compounds were detected from different day culture fractions of *Frankia* using GC-MS-MS. A total of 19, 23 and 14 components were identified and varied in different day's cultures respectively. Hexa decanoic acid, Phthalic acid and its ester derivatives are present in 25th and 30th day culture. The terpene derivatives (Hopanoids) Viminalol and β-Amyrine are present in 15th day cultures found essential for nodulation and nitrogen fixation in *C. equisetifolia.* The *Frankia* are known to contain a large percentage of hopanoids are essentially produced under nitrogen-fixing conditions stabilize the walls to keep oxygen away from the nitrogenase [3]. The hopanoids themselves play a more specific and molecular role in the oxygen protection mechanism of nitrogenous [26]. Phenol,2,4-bis(1,1-dimethylethyl) is the only compound identified in both 15th & 25th day culture but lacking in 30th day culture, whereas all other compounds identified are unique and present either any one of the culture. The bioactive compounds isoterpinolene, 2, 4, Phenol-bis (1, 1-dimethyl ethyl) and 1-Dotriocantanol were eluted at Rt 12.468, 18.126 19.105 mt. reported to have free radical scavenging activity capable of protecting the cells from oxidative stress [27]. Since oxygen inactivate nitrogenase activity, the plant produces those compounds having high affinity to oxygen originated around the root prevent it to reach the nitrogenous complex. *Frankia* has produced a complex of bioactive compounds, such as hoponoids and terpenoids at different stages during its growth period, and at 15th day from the date of *Frankia* inoculation reported to produce

vesicles in roots of *C.equisetifolia*. However the compounds such as isoterpinolene, 2, 4, Phenol-bis (1, 1-dimethyl ethyl) and 1- Dotriocantanol are produced in later stages of *Frankia* growth period tend to restrict the oxygen disturbances in the nitrogenous activity, thereby enhancing nodulation and nitrogen fixation in *C. equisetifolia.*

Growth and Biomass of Frankia inoculants treated *C. equisetifolia* rooted cuttings: Microbial inoculation resulted in significant increase in plant height, nodule number and nodule weight of *C.equisetifolia* cuttings treated with *Frankia* culture, compared to the uninoculated control and crushed root nodules (experiments conducted at IFGTB nursery). Nodulation was observed at 25th day in the *C. equisetifolia* seedlings inoculated with *Frankia* culture. The initial infections at 20th days showed clubbed roots in the rooted stem cuttings, and the nodule development occurred at 25th day. Survival of *C. equisetifolia* cuttings was 100% in all the treatments compared to the uninoculated control in which survival is only 70 %. It has been proved that providing *Frankia* inoculums in early stage of plant development is an important aspect advocated for quality planting stock productivity of casuarinas [28]. Several investigators have reported that dual inoculation of AM fungi and *Frankia* resulted in better growth of actinorhizal plants compared to individual inoculation [29]. Within the inoculants, 15th day *Frankia* culture induced significant increase in plant height compared to other inoculants, control and crushed root nodules due to the presence of mono-sesquiterpenpids, fatty acid and hopanoid derivatives etc., (Table 2 & 3; Fig. 1). Similarly, Reddell [30] and Rajendran *et al.* [31] showed that artificial application of nodule crush increased dry matter yield of casuarina. The increase in height of the *C.equisetifolia* cuttings after co-inoculation might be caused by the improved accumulation of nitrogen due to nitrogen fixation by *Frankia* [32]. Nodule number and nodule weight was high with 25th day and 30th day *Frankia* culture compared to other inoculants as reported by Rajendran and Devaraj [33], for instance have found via field inoculation studies that the biomass of *C. equisetifolia* inoculated with *Frankia* is higher than that of the non-inoculated control.

Biochemical profiles of *C.equisetifolia* seedlings treated with Frankia inoculants: The major soluble fractions of nitrogen pool of the nodulated plants releases the ureides, allantoin and allantoic acid throughout vegetative and reproductive growth. In the case of *C. equisetifolia*, seedlings treated with 25th day *Frankia* culture expressed the allantoin content which was profoundly high as compared to 15th, 30th day culture and crushed root nodules, and more for the inoculants treated seedlings. The similar finding was reported in soybean, (*G. max*) nodule is the presumed site of synthesis of ureides [23]. The labeling studies using $^{14}C0_2$ and $^{15}N_2$ in cowpea indicated that synthesis of allantoin and allantoic acid in root nodules involved photosynthate and fixed N, and that the ureides were exported from nodule to shoot via the xylem [34]. The shoot metabolized more than 80% of the ureides which acted as major N sources for protein in all the growth periods. Glutamate dehydrogenase content was high in 30th day *Frankia* culture treated *C. equisetifolia* seedlings, on the contrary, it was observed that the glutamate synthase activity was higher in 25th day *Frankia* inoculants treated seedlings *C.equisetifolia*. This was demonstrated by Miflin and Lea [35] that extracts of the plant tissue fraction of nitrogen-fixing cowpea nodules contained glutamate synthase and glutamine synthetase raises the possibility of ammonia formed in N fixation by bacteroids might be incorporated by the host cytoplasm in a manner similar to the mechanism proposed for amide-producing species. Microbial inoculants and crushed root nodules increased the free amino acid content in *C.equisetifolia* seedling treated with crushed root nodule 15th,

30th and 25th day *Frankia* inoculants (Table 4). The increases in nitrogen content are due to nodulation and nitrogen fixation as well as to symbiotic association of *Frankia* with casuarinas.

Biomass documentation of the *C.equisetifolia* seedlings treated identified biosignalling compounds: Biosignalling compounds having oxygen quenching activity, which is responsible for nodulation and nitrogen fixation found to induce root nodules profoundly and biomass in *C.equisetifolia* seedlings. Enhanced root nodules, nodule weight and plant height were comparatively higher in the treated seedlings due to the presence of monophenols which is only one hydroxyl group as reported by Abd-Alla [36] in soybean. Wain and Taylor [37] reported that the presence of two OH groups at the ortho position was necessary for the growth promotory activity of phenols. The presence individual phenol and phenolics like catechin, Phenyl acetic acid, phthalic acid etc. were found to induce increases in number of nodules when compared to other active compounds which induced only one within 25 days of inoculation, where as in control no root nodulation was occurred. Crushed root nodules induced very limited response of inducing root nodule which indicated that biosignalling compound treatment could be a better alternate to induce nodulation in casuarinas when compared to other products. *Frankia* culture establishment is time consuming as pure *Frankia* culture establishment is difficult task to achieve. Among the various tested concentrations of phthalic acid, catechin, epicatechin and phenyl acetic acid at 100-1000µM, the plant height found to increase and recorded maximum as compared to control. In the case of phthalic acid treatment, the plant height was was maximum for seedlings treated with 100 µM. Catechin treated seedling attained significant height at 250 µM-1000µM. Epicatechin treated seedling showed decrease in plant height with increase in concentration. Overall among the four bioactive compounds tested catechin at 1000µM showed maximum plant height. Nodule weight was also more in the all the treated seedling may be due to the induction of phenols, mono-, di-and tri-derivatives each at concentrations of 100 µM significantly increased nodulation, plant dry matter and total plant nitrogen in soybean when applied with the irrigation solution [36]. Biosignalling compounds identified from *Frankia* culture chromatographically, found to increase biomass significantly due to casuarina- *Frankia* symbiotic association. The above results indicated the biomass production of casuarina treated with the bioactive compounds have significantly influenced the plant height, nodule number and nodule weight casuarinas instead of *Frankia* treatment (Fig. 2-4).

Biochemical documentation of the *C.equisetifolia* seedlings treated with identified biosignalling compounds: Allantoin content was high for the nodules of *C.equisetifolia* seedlings treated with phenyl acetic acid (0.392 mg/g) followed by phthalic acid (0.354 mg/g), epicatechin (0.168 mg/g) and Catechin (0.133 mg/g) respectively. Allantoin content was profoundly increased in all the bioactive compound treated *C.equisetifolia* seedlings nodules than control. Glutamate dehydrogenase (2.257 mg/g) and Glutamate synthase content (1.504 mg/g) was also high for phenyl acetic acid treated *C.equisetifolia* seedlings. In the nodules, the assimilation of ammonia into amino acids occurs primarily via glutamine synthetase and glutamate synthase and both the enzymes were increased in the treated seedlings, which indicate the role of those chemicals for the variation of nitrogen content due to *Frankia* inoculation [19]. Glutamine synthetase (GS), a key enzyme for root nodule metabolism, is a molecular target of NO in root nodules of *Medicago truncatula*, being regulated by tyrosine (Tyr) nitration in relation to active nitrogen fixation [38]. Free amino acid content was also high in phenyl acetic acid treated seedlings than all bioactive compounds and crushed root nodule inoculated seedling. Free amino acid quantity increased in treated seedlings than control. Phenyl acetic acid provides better induction in terms of

increase in nitrogen content due to nodulation and nitrogen fixation, compare to other bioactive compounds (Fig. 5).

CONCLUSION:

Frankia as a source of bioactive compounds has largely gone uncharacterized has been elucidated from different day cultures of a root endophyte as hopanoid derivatives. Hopanoids are intuitive secondary biosignalling molecules of *Frankia* which are nitrogen fixing an actinomycete symbionts induce the formation of root nodules of a diverse group of plant species. It has been observed from the present investigation is that some of the bioactive secondary molecules are more prominent factors for establishing the root-associated growth of *Frankia* due to the presence/ variations in the presence of biotransformed signaling molecules, especially the hexa decanoic acid, phthalic acid and their derivatives at different stages of its growth period of nodulated *C.equisetifolia*. In the present study, it is found that the *Frankia* has produced a complex of bioactive compounds, such as hoponoids and terpenoids and their role in plant-pathogen and plant-insect interactions is receiving increasing attention because of their potential use in pest control, however, little is known about their physiology and the regulation processes of the plant-endophyte interaction especially during sporulation and the bioconversion of bioactive compounds for nodulation capacity with multiple applications in casuarinas.

Table 1: Metabolite quantification of Frankia culture and *Casuarina equisetifolia*

Secondary metabolites and enzymes	Frankia culture			Casuarina	
	15th day	25th day	30th day	Root	Root nodules
Protein (mg/ml)	0.001	0.0016	0.0023	3.64	3.9
Carbohydrate (µg/ml)	0.322	1.076	0.050	1.433	1.043
Lipid (mg/g)	1.122	1.571	2.244	3.366	5.61
Phenol (µg/ml)	0.107	0.028	0.124	1.68	1.69
Phenolics (µg/ml)	0.186	0.432	0.269	1.453	1.449
Flavanoids (µg/ml)	0.036	0.041	0.027	0.774	0.463
Allantoin (µg/ml)	0.153	0.273	0.187	0.127	0.261
Free amino acids (µg/ml)	5.625	3.625	5.00	0.625	1.125
Glutamate dehydrogenase (µg/ml)	0.968	1.577	1.823	1.10	1.88
Glutamate synthase (µg/ml)	0.936	3.042	1.093	1.40	2.44

Table 2: GC-MS/MS profile of different day Frankia culture for casuarina biomass

Retention time	Name of the compound	Area	Height	15th day	25th day	30th day
5.548	3,4-dimethoxy-1-pentene	6.775	3.885	-	✓	-
11.535	Benzaldehyde,2,4-dimethyl	4.977	1.270	-	✓	-
12.468	Isoterpinolene	7.547	2.527	-	✓	-
13.220	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)	7.512	3.023	✓	-	-

13.619	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)	6.884	3.033	✓	-	-
13.677	Benzene acetic acid, α-methoxy methyl ester	4.017	1.474	-	✓	-
16.945	Cyclohepta siloxane, tetradecamethyl	4.679	1.156	-	✓	-
18.126	Phenol,2,4-bis(1,1-dimethylethyl)-CAS	4.167	1.551	✓	-	-
18.246	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)	4.002	1.377	✓	-	-
18.472	1,2,3,5 tetra methyl cyclohexane (1R,2C,3C)	4.257	1.558	✓	-	-
18.647	1-Detriacontanol	7.045	2.014	✓	-	-
18.879	Isotrideconol	5.953	2.156	✓	-	-
18.979	2,6-di-(t-butyl-4-hydroxy-4-methyl)-2,5-cyclohexadiene-1-one	4.118	1.538	✓	-	-
19.105	1-Detriacontanol	4.127	1.469	✓	-	
20.027	3-n-Hexyltriolane,s,s-dioxide	4.110	1.450	✓	-	-
25.768	Phthalic acid, pentyltridex-2-yn-1-yl-ester	8.529	3.445	-	✓	-
25.770	(3R,4S)-3-(2-Notro-4-methoxy phenyl)-4-(4-hydroxy phenyl) hexane	5.714	2.364	-	-	✓
26.451	2,3,5,8-tetrahydroxy-6-methyl anphthol-1-4-puinone	6.983	2.704	-	✓	-
27.641	Phthalic acid, butyl-2-pentyl ester	5.754	2.121	-	-	✓
27.645	Phthalic acid, pentyltridex-2-yn-1-yl-ester	4.086	4.568	-	✓	-
27.708	Hexa decanoic acid	4.020	1.451	-	-	✓
28.622	Phenol-2-[(4-hydroxy phenylmethyl-)]	6.271	2.081	-	✓	-
29.399	Quinolphos	4.840	1.172	✓	-	-
29.866	Phenol-(4,4-methylene bis-)CAS	6.718	2.113	-	✓	-
30.264	2,3, Dihydroxy-propyl elaidate	5.053	1.961	-	✓	-
36.690	Phenol-2,4-bis-(1-phenlyethyl)-	5.455	1.595	-	✓	-
37.418	2,Beta-(3-Oxobutyl-1-α-3,3-trime)	4.408	1.440	-	✓	-
41.377	1,6,10,14,18,22-tetracosa hexane-3-ol-2	7.851	2.773	✓	-	-
43.111	Beta-Amyrin	8.575	2.936	✓		
44.172	Viminalol	9.437	2.882	✓	-	-

Table 3: *Casuarina equisetifolia* cuttings (6 month old) treated with Frankia Culture

Treatment	**Increase in plant height (cm)**	**No of nodules**	**Nodule weight (gm)**
Control	2.3 ± 1.62	1	0.42
15th day culture	3.08 ± 2.18	1	0.50
25th day culture	2.86 ± 2.02	2-3	0.67
30th day culture	2.36 ± 1.67	2-3	0.70
Crushed root nodule	2.42 ± 1.22	1	0.60

Table 4: Biochemical documentation of the *C.equisetifolia* seedlings treated with Frankia inoculants

Material	Allantoin content (mg/g)	Glutamate dehydrogenase (mg/g)	Glutamate synthase (mg/g)	Free amino acids (µg/mg)
Control root	0.127	1.10	1.40	4.000
15th day *Frankia* culture treated root nodule	0.140	0.968	0.936	5.625
25th day *Frankia* culture treated root nodule	0.223	1.577	3.042	4.625
30th day *Frankia* culture treated root nodule	0.163	1.823	1.093	5.00
Crushed root nodule treated root nodule	0.180	1.88	1.128	6.125

Fig. 1: *C.equisetifolia* cuttings treated with Frankia culture

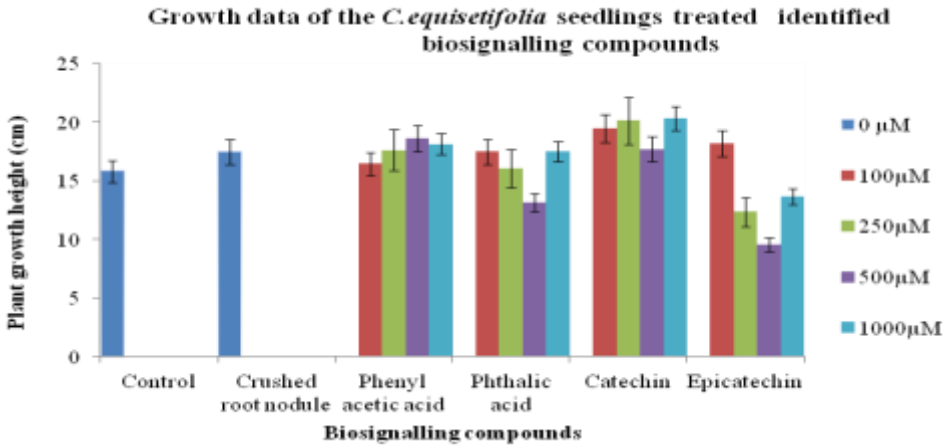

Fig. 2: Growth data of the *C.equisetifolia* seedlings treated with identified biosignalling compounds

Fig. 3: Root nodules in *C.equisetifolia* seedlings treated with biosignalling molecules

Fig. 4: *C.equisetifolia* seedlings treated with identified biosignalling compounds

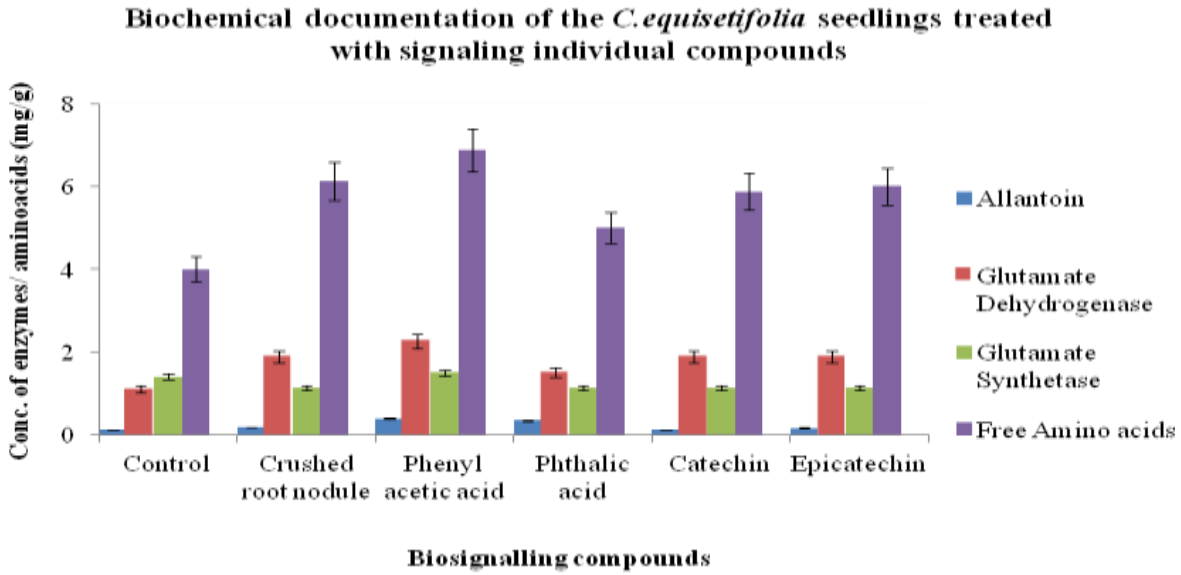

Fig. 5: Biochemical documentation of the *C.equisetifolia* seedlings treated with signaling individual compounds

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Corresponding Author: S. Murugesan

Division of Bioprospecting, Institute of Forest Genetics & Tree Breeding, Coimbatore, Tamilnadu, India.