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Arbuscular mycorrhiza influences the growth and biochemical parameters of *Cassia fistula* L. seedlings, contrasting with the naturally occurring established trees

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Abstract

The efficacy of rhizospheric native arbuscular mycorrhizal fungi from naturally growing *Cassia fistula L.* trees was evaluated by utilizing seeds from the same trees to assess growth and biochemical properties. This study aimed to investigate whether biochemical content could be augmented in naturally growing trees, contrasting them with greenhouse-grown seedlings. The findings revealed that arbuscular mycorrhiza-inoculated seedlings exhibited significantly higher shoot and root length, leaf area, and shoot dry weight compared to non-mycorrhizal plants under greenhouse conditions. Furthermore, there was a noteworthy increase in biochemicals such as protein, carbohydrates, and phenols in mycorrhizal-inoculated plants and naturally growing trees when compared to non-mycorrhizal plants. Interestingly, carbohydrates and phenols were significantly more abundant in naturally growing trees than in greenhouse experiment plants. Additionally, the methanolic leaf extract of non-mycorrhizal plants exhibited the lowest inhibition percentage (%) on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals in comparison to mycorrhizal plants and naturally growing trees. Moreover, the effective concentration at 50% inhibition of DPPH radicals by mycorrhizal plants and naturally growing trees was lower than that observed in non-mycorrhizal plants. The biochemical estimates obtained from mycorrhizal plants substantiate the results observed in naturally growing trees, thereby supporting the assumption that native arbuscular mycorrhizal fungi may enhance the levels of biochemicals in naturally growing *C. fistula* trees.

Key words: AM fungi, *Cassia fistula*, growth features, biochemicals, antioxidant activity

Introduction

The association of plants with arbuscular mycorrhizal (AM) fungi is a prevalent phenomenon, with over 70 percent of plants capable of forming such interactions (Smith and Read, 2008; Brundrett, 2009). This symbiosis involves fungi from the *Glomeromycota phylum* (Tedersoo *et al.*, 2018), wherein the fungus acquires carbon from the host while aiding in the plant's uptake of phosphorus (P), nitrogen (N), and other mineral nutrients from nutrient-deficient soil (Alimi, 2021). Known for their ability to improve soil structure and enhance plant performance, AM fungi contribute to the establishment, resilience to environmental stresses, and increased nutrient and water uptake by plants (Jeffries and Barea, 2012).

The significance of AM fungi in promoting plant growth has been extensively documented (Duponnois *et al.*, 2005; Ghosh and Verma, 2006; Ndoye *et al.*, 2013; Chen *et al.*, 2021). Studies have also explored the impact of AM fungi on the augmentation of plant biomolecules (Oliveira *et al.*, 2013; Lima *et al.*, 2015, 2017; da Silva *et al.*, 2018), with attention turning toward the potential pharmaceutical applications of bioactive molecules found in mycorrhiza-inoculated plants (Oliveira *et al.*, 2013).

Previous research has investigated the mycorrhizal status of various *Cassia* species, including *C. absus, C. auriculata, C. fistula, C. hirsute, C. occidentalis, C. pumila* and *C. tora* (Muthukumar and Udaiyan, 2000), as well as *C. grandis, C. siamea*, and *C. spectabilis* (Wang and Qiu, 2006). Studies on *C. fruticosa* seedlings and other tropical rainforest species have confirmed their mycotrophic nature, with growth promotion attributed to mycorrhizal associations (Janos, 1980). Additionally, AM fungi have been reported to enhance the growth performance of *C. siamea* in semi-arid Indian wasteland soil (Giri *et al.*, 2005).

C. fistula, a moderately sized deciduous tree belonging to the Caesalpiniaceae family, is known for its medicinal properties, bearing yellow flowers in lax racemes (Deb, 1981). The plant exhibits diverse pharmacological activities, including analgesic, anti-inflammatory, antioxidant, hepatoprotective, anticancer, and antiulcer effects (Ilavarasan *et al.*, 2005; Karthikeyan & Gobianand 2010; Sheikh *et al.*, 2010; Irshad *et al.*, 2014).

In this study, we have attempted to use the seeds from mother plants to assess the efficacy of the native mycorrhizal consortium associated with the same plant. Explicitly we have employed both the indigenous mycorrhizal inoculum collected from the vicinity of the growing tree and inoculated the germinated seeds of *C. fistula* of the same mother tree. The selection of *C. fistula* was driven by its medicinal importance. This study aims to examine the growth characteristics and biochemical properties of *C. fistula* seedlings under greenhouse conditions. Additionally, we assessed the antioxidant and biochemical properties of fully grown mature trees in their natural environment to corroborate results obtained from greenhouse plants. This approach seeks to support the hypothesis that native mycorrhiza enhances the biochemical profile of plants growing under natural conditions.

Materials and methods

Plant materials: The study was conducted at Tripura University Campus in Suryamaninagar, Tripura, Northeast India (23º45.895N; 091º15.618E, 64 masl), covering an area of 79 acres (31.9702 ha) with diverse vegetation, including naturally growing patches of *Cassia fistula* (NGT), *Acacia auriculiformis*, *Michelia champaca*, *Anacardium occidentale*, *Polyalthia longifolia*, and others (Deb *et al.*, 2016). The mother trees of *C. fistula* in the NGT area exhibited heights of 7-9 m and diameters at breast height (DBH) of 40-42 cm. For the greenhouse experiment, twenty matured black-colored pods (18-20 cm in length) were collected from a single NGT plant.

Inoculum used: The mycorrhizal inoculum was obtained from the rhizospheric zone of three NGT trees, containing mycorrhizal species such as *Glomus multicaule* Gerd. & Bakshi, *Glomus heterosporum* Sm. & Schenck, *Glomus hoi* (Nicolson & Gerd.) Walker & Schüßler, *Gigaspora margarita* Becker & Hall, and *Rhizophagus diaphanus* (Cano & Dalpé) Walker & Schüßler. AM fungal propagules from the same trees were used as the mycorrhizal inoculum for greenhouse experiments to maintain uniformity and equal spore density. The spore density in the soil was 317±31 spores/100 g soil.

Experimental conditions: Soil was collected from a depth of 20 cm around three mother trees of *C. fistula*. The soil underwent two rounds of autoclave sterilization and was used for both mycorrhizal (AM+) and non-mycorrhizal (NM-) treatments. The AM+ treatment involved direct inoculation of the sterilized soil with native inoculum, while the NM- treatment used sterilized soil without AM propagules. The germination and propagation of seedlings were carried out using seeds from mature pods. The experiment was set up with fifty seeds in each treatment, planted one cm below the soil surface. The germination percentages for AM+ and NM- were 62 and 36%, respectively, with seedling survival percentages of 30 and 10%. Soil parameters included organic carbon (0.59%), available phosphorus (1.91 mg/g), and total nitrogen (27.72 kg/ha).

Determination of growth characteristics: After 90 days of germination, various growth characteristics, including shoot length, root length, shoot dry weight, root dry weight, and leaf area, were assessed from three plants. Plant dry weight was determined after drying the tissues in an oven at 70°C for 48h, and leaf area was measured using graph paper.

Estimation of mycorrhizal colonization: Mycorrhizal colonization was quantified using roots collected from three NGT trees and from three seedlings in both AM+ and NM- treatments after 90 days of inoculation. Roots were stained according to Das and Kayang (2008), and mycorrhizal colonization was determined under a bright field microscope (Olympus CX21i) using the magnified intersection method (McGonigle *et al.*, 1990).

Biochemical estimation: Biochemical analyses, including protein and carbohydrate assessments, were conducted after 90 days of inoculation using the methods of Sadasivam and Manickam (1996). Phenolic concentrations in methanolic extracts were determined as per Ferreira *et al.* (2007), and all analyses were performed spectrophotometrically (UV-VIS Biospectrometer, Eppendorf).

Antioxidant activity: For antioxidant activity, methanolic leaf extracts from NM-, AM+, and NGT samples were obtained following the modified method of Roy Das *et al.* (2017). The extracts were evaluated for their effect on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical using the method of Kirby and Schmidt (1997). Inhibition percentages were calculated, and the concentration providing 50% inhibition (EC50) was determined.

Data Analysis: Results were expressed as mean ± SE, and analysis of variance (ANOVA) was conducted. Means were separated by Duncan test at $P < 0.05$. All the data analyses were performed using Statistica ver. 9.0 (Statsoft).

Result and discussion

Effect of native mycorrhiza on *C. fistula* seedlings: The greenhouse experiment, utilizing seeds from naturally growing *C. fistula* (NGT), aimed to evaluate the efficacy of native mycorrhiza on the growth and biochemical properties of the seedlings. The results demonstrated a significant positive influence of mycorrhiza in AM+ compared to NM-. Biochemical analysis in AM+ further substantiated the findings when compared with NGT, supporting the hypothesis that native mycorrhiza may enhance the biochemical content of NGT.

Mycorrhizal colonization and growth properties: Mycorrhizal colonization percentages for AM+ and NGT were comparable, with AM+ exhibiting a slightly higher colonization percentage. No colonization was observed in NM- plants. The growth analysis revealed that shoot and root length, leaf area, and shoot dry weight were significantly higher in AM+ compared to NM-, indicating a substantial influence of mycorrhizal inoculation on *C. fistula* seedlings' growth (Table 1). However, there was no significant difference in root dry weight between AM+ and NM-.

Table 1. Growth properties with mean in mycorrhizal (AM+) and non-mycorrhizal plants (NM-) of *Cassia fistula* seedlings

Treatments	NM-	AM+
Shoot length (cm)	17.50±0.29b	21.50±1.15a
Root length (cm)	16.67±1.86b	29.33±1.17a
Leaf area (cm^2)	6.51±1.68b	19.76±2.62a
Shoot dry weight (g)	0.27±0.03b	1.95±0.36a
Root dry weight (g)	0.11±0.05a	0.90±0.34a

Means followed by the same letter do not differ significantly at $P < 0.05$ according to Duncan test.

The findings align with previous studies indicating that mycorrhizal root colonization and plant growth are enhanced in sterilized soils, regardless of soil origin and AM isolates (Ndoye *et al.*, 2013). AM fungi play a crucial role in improving plant growth by increasing nutrient uptake, water absorption, and the transport and absorption of inorganic nutrients, particularly phosphorus (P) (Sun and Shahrajabian, 2023). The observed increase in shoot length, root length, shoot dry weight, and leaf area supports this, and similar effects were noted in other studies involving different plant species (Gaur and Adholeya, 2002). Many times, it is suggested that genetic variations affecting the degree of root colonization could dictate differences in the growth response to AM since more arbuscules could provide the plant with more

Fig. 2: Growth of *Cassia fistula* in non-mycorrhizal (left) and mycorrhizal condition (right).

mineral nutrients (Berger and Gutjahr, 2021). The genotypes of the plant and the fungus, in addition to the environmental factors, determine the AM responsiveness (Berger and Gutjahr, 2021).

Biochemical characteristics: Biochemical analysis revealed a significant increase in protein, carbohydrate, and phenol content in AM+ plants compared to NM- plants. However, the biochemical parameters, except for protein, were significantly higher in NGT compared to greenhouse experiment plants. The elevated biochemical properties in AM+ plants are consistent with previous studies (Oliveira *et al.*, 2013; Lima *et al.*, 2015). The enhanced nutrient uptake by plants in AM+ possibly contributes to the observed increase in biochemicals. This suggests that AM-mediated plants may accumulate higher phenolics, supporting earlier reports (Lima *et al.*, 2017). Phenolics, known to act as protective agents against various stresses, were higher in NGT, although the leaves collected were healthy and free from infections. Moreover, NGT growing in natural environment coping with unseen biotic and abiotic stress, thereby having increased levels of phenolics and proteins, thus, supporting that AM responsiveness may lead to accumulation of transcripts, proteins or secondary metabolites, which may for instance bear nutritional or medicinal value (Berger and Gutjahr, 2021).

Antioxidant activity: Antioxidant activity assessment using DPPH radicals revealed that leaves of NM- plants exhibited the lowest inhibition percentage compared to AM+ and NGT plants. NGT leaves had a higher inhibition percentage than the other plant samples, and at various concentrations, NGT and AM+ plants did not differ significantly. The EC50 values of NM- plants were lower than the standard and the other treated samples,

Table 2. Biochemical properties, effective concentration at 50 % inhibition of DPPH radicals (EC_{50}) and mycorrhizal colonization in mycorrhizal (AM+), non-mycorrhizal plants (NM-) and naturally growing trees (NGT) of *Cassia fistula*

Treatments	NM-	AM+	NGT
Protein (mg/g leaf fresh wt.)	18.69±0.35b	26.23±0.13a	27.07±0.48a
Carbohydrate (mg/g leaf fresh wt.)	26.50±0.07c	46.10±0.21b	61.83±2.45a
Phenol (mg/g leaf fresh wt.)	6.37±0.05c	17.23±0.06b	18.04±0.23a
EC50	0.57	0.51	0.51
Mycorrhizal colonization (%)	0	69.15±3.89a	62.70±5.48a

Means followed by the same letter do not differ significantly at $P < 0.05$

indicating that mycorrhizal inoculation significantly influenced the antioxidant activity of leaf extracts from *C. fistula* seedlings.

The enhancement of antioxidant activity by mycorrhiza in *C. fistula* aligns with previous studies (Rozpądek *et al.*, 2014; Haghighi *et al.*, 2016; Avio *et al.*, 2017).

The comparison between seedlings and naturally growing trees was challenging due to inherent differences existed in age, root system development, and trees growing in natural environmental condition with little attention to management. The degree of mycorrhizal colonization, spore density, and glomalin-related soil protein were all significantly impacted by tree age (Han *et al.*, 2023), nevertheless, the mycorrhizal colonization in the present study does not differ significantly. The spore density of young olive rhizosphere was found to be significantly higher than that of old plantations (Meddad-Hamza *et al.*, 2017), however, in our study the spore propagules were inoculated in the greenhouse plants equally as the same density of spores extracted from the rhizosphere of NGT. Mature tree litter returns nutrients to the soil, which helps to explain why the soil has a high potassium content (Han *et al.*, 2023) since seedlings grown in the greenhouse conditions of the current study were adequately maintained to prevent litter from developing into nutrients. Although the above-mentioned differences and certain commonalities existed in NGT as compared to seedlings in greenhouse condition, the present study was an effort to compare only the biomolecules in both the conditions and provides the basis of experiment of AM fungal amelioration effect.

Taking into account the particular conditions *viz*., observed in the case of seedlings which were in growing peaks as compared to trees reaching the maximum growth condition in the present investigation. To be specific the carbon cost of maintaining the microsymbiont may outweigh the benefits of mycorrhization, it can be assumed that excessive colonization by the fungus was not beneficial for the plant at certain developmental stages (Shtark *et al.*, 2019). The dynamics of AM development were not tracked in the current study during the plant development. As previously noted, the growth curve may rise until the plants flower and at that point, the fungi cease to develop and simply need carbon for maintenance (Shtark *et al.*, 2019) as in the present work the same situation could be attributed to NGT. However, because the root system is small and hyphae are more effective at accessing P, which is weakly mobile in soil, the relative costs of AM symbiosis are higher in the

Table 3. Inhibition percentage of methanolic extract of leaves of non-mycorrhizal plant (NM), arbuscular mycorrhizal (AM) inoculated plants and natural growing trees (NGT).

Concentration (mg/mL)	BHA	Aa	NM	AM	NGT
1	96.72±0.10a	93.19±0.72cd	85.33±2.90f	93.97±0.69cd	95.02±0.17b
2	95.70±0.10b	95.75±0.48b	94.67±0.07c	96.35±0.02a	95.76±0.08 b
4	94.40±0.19c	97.20±0.19e	93.49±0.11cd	95.11±0.25b	96.53±0.28 a
8	93.20±0.15cd	92.81±0.05d	89.98±0.18g	92.69±0.37d	98.67±0.12e
16	90.54±0.34e	97.01±0.05de	85.58±0.15f	89.21±0.52g	99.02±0.04he

Means followed by the same letter do not differ significantly at $P < 0.05$ according to Duncan test. BHA-Butylated hydroxyanisole, Aa-Ascorbic acid early stages of plant development when AM fungal colonization is essential for plants (Kaschuk *et al.*, 2009) as the seedlings was in its highest growing peak as compared to NGT.

In conclusion, the use of native mycorrhizal inoculum emerges as a valuable biotechnological tool for producing *C. fistula* seedlings with a substantial accumulation of biomolecules. This enhancement holds great promise for the pharmaceutical industry, afforestation programs, and horticultural applications. The study lays the groundwork for a comparative analysis supporting the benefits of AM fungi in NGT concerning biochemical concentration, which can be extrapolated to other naturally growing tree species. The limitation stands in the form of the molecular analyses through affiliation of AM taxa residing in NGT as well as in greenhouse plants to further validate the existence of native inoculum in both the conditions. To add another constraint is the effect of AM symbiosis on the growth of natural trees as comparison with the seedlings does not hold the true scenario where the natural condition may include the developed plant in stress period as well as the role of AM on the physiology and the developmental stage which is different in terms of its age. Future research could explore comparisons with herbaceous species and/or annuals in natural conditions, evaluating morphometric and biochemical aspects between AM+ and NGT, further highlighting the positive effects of AM fungi and simultaneously validating through molecular analysis from environmental samples.

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