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Quantitative estimation of amino acids and carbohydrates present in the root exudates of *Suaeda nudiflora* and *Cassia auriculata*

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Abstract
Substances released by healthy and intact roots into the environment are collectively designated as root exudates. In present study, *Suaeda nudiflora Moq.* and *Cassia auriculata* Linn. were selected as experimental plants. *S. nudiflora*, a halophytic species; it has the potential as a future oil seed crop and is highly suitable for producing high protein biomass while *C. auriculata* is used as fodder for camels and goats and also known for its various medicinal properties like anthelminthic, antidiabetic, etc. Root exudates were collected from both the plants and carbohydrate and amino acids were measured quantitatively. Protein/amino acids estimation were done by the methods Ninhydrin and Bradford's colorimetric assay while carbohydrate estimation was done by the methods Anthrone and Phenol sulfuric Acid colorimetric assay. Result of this study showed that *C. auriculata* was showing better result than *S. nudiflora* in Ninhydrin, Bradford's and Phenol-sulfuric Acid colorimetric assays while *S. nudiflora* was showing better result than *C. auriculata* in Anthrone colorimetric assay. In the rhizosphere, root exudate plays an important role as attracting signal molecules to attract the plant growth promoting microbes (endophytes and rhizobacteria) which performs important plant growth promoting functions (biocontrol, hormone production and bioremediation). Experimental plants showed the presence carbohydrate and amino acids in root exudates, which will help them in the promotion of plant growth, immunity and increase the resistance to arid and saline conditions which is not only of economic importance but also counted as contribution to the agriculture field.

Keywords: *Suaeda nudiflora*, *Cassia auriculata*, Root exudates, Amino acids, Carbohydrates, Colorimetric assay

1. Introduction
Substances released by healthy and intact roots into the environment are collectively designated as root exudates. Many compounds are released by plant roots, including inorganic ions and substances, amino acids, amino acids, amides, sugars, aliphatic acids, aromatic acids, volatile aromatic compounds, gases (Vančura, 1988; Grayston *et al*., 1996; Paynel *et al*., 2001; Aulakh *et al*., 2001; Uren, 2007; Neumann and Romheld, 2007) [60, 19, 41, 2, 58, 38]. The rhizosphere is richly populated with microorganisms that subsist on compounds released from plant roots. These microbes, in turn, can enhance uptake of nutrients by plant roots and enable uptake of otherwise unavailable nutrients from the soil (Lugtenberg and Kamilova, 2009) [32]. Rhizosphere-dwelling microbes can induce systemic disease resistance in plants, suppress colonization of root pathogens, and stimulate endophytic colonization (Lugtenberg and Kamilova, 2009) [32]. Of these amino acids and carbohydrates are the two important substances that are released in the root exudates as they play many crucial roles including disease suppression (Haas and Defago 2005; Mendes *et al*. 2011; Weller *et al*. 2002) [20, 35, 64], positive plant–microbe interactions (plant-growth promoting bacteria like endophytes and rhizobacteria), vital ecosystem processes such as carbon sequestration and nutrient cycling (Singh *et al*. 2004) [50], increased nutrient availability and uptake (Lugtenberg *et al*. 2002; Morrissey *et al*. 2004) [33, 37], and increased immunity to abiotic (Selvakumar *et al*. 2012; Zolla *et al*. 2013) [47, 70] and biotic stresses (Badri *et al*. 2013*b*; Zamioudis and Pieterse 2012) [4, 69], each of which leads to increases in plant productivity (Berg 2009). In turn, the plant provides the soil microbes with root exudates that are used as substrates and signaling molecules (Bais *et al*. 2006) [5].

Amino acids are ubiquitous in biological systems as the functional units in proteins and are also shown to alter key phenotypes related to plant root growth and microbial colonization, symbiotic interactions, and pathogenesis in the rhizosphere (Moe, 2013) [36]. Chemotaxis toward root exudates is a well-appreciated trait of rhizosphere colonizing bacteria that is essential for root colonization (de Weert *et al*., 2002) [14]. Amino acids play a significant role in synthesis and regulation of the activity of the auxin phytohormones (Staswick, 2009) [54], which function in myriad plant processes including root development and architecture (Woodward and Bartel, 2005) [65]. Soil-dwelling microbe's chemotax toward amino acids readily, more so than to other root exudate components, and the presence of free amino acids in the rhizosphere likely contributes to the richness of the microbial community at the plant root–soil interface (Moe, 2013) [36].

Recent work has explored the roles of amino acids in biofilm formation and disassembly among both Gram-positive and Gram negative rhizosphere-dwelling bacteria (O'Toole and Kolter, 1998; Valle *et al*., 2008; Kolodkin-Gal *et al*., 2010) [40, 59, 26]. Amino acids are now appreciated as a key intermediary in the soil nitrogen cycle and generation of ammonium from amino acids in soil is carried out by extracellular amino acid oxidases (Moe, 2013) [36]. Amino acids serve as a key mobilizable source of nitrogen in plants, and their transport across cell membranes is necessary not only for uptake from soil but also for long-distance transport from points of synthesis or uptake through the phloem to various developing tissues (Moe, 2013) [36].

Increasing interest is devoted to carbohydrates for their roles in plant immunity. Some of them are elicitors of plant defenses whereas other ones act as signaling molecules in a manner similar to phytohormones (Trouvelot *et al*., 2014) [56]. Additionally, it has a pivotal function as signaling molecules, in a manner similar to hormones, has become apparent (Koch, 1996, 2004; Sheen *et al*., 1999; Rolland *et al*., 2006; Smeekens *et al*., 2010) [24, 25, 48, 44, 52] and is nowadays largely investigated. Hence, as they interact with diurnal changes, abiotic and biotic stresses, and hormone signaling, sugars are considered as actors of a complex communication system necessary for the coordination of metabolism with growth, development, and responses to environmental changes and stresses (Rolland *et al*., 2002, 2006) [45, 44]. In addition, there is growing evidence for a role of sugars as antioxidants as they possess ROS scavenging properties. Sugars could therefore be considered as key components of an integrated cellular redox network, as this role was recently reviewed in details by Keunen *et al*. (2013) [22]. In plant microbe interactions, sugars are essential to fuel the energy required for defenses and serve as signals for the regulation of defense genes (Ehness *et al*., 1997; Roitsch *et al*., 2003; Bolton, 2009) [16, 43, 9]. The potential key roles of some sugars regarding plant immunity have recently led to the "sweet Immunity" and "sugar-enhanced defense" concepts (Bolouri-Moghaddam and Van Den Ende, 2013) [8]. On knowing their important roles in plant growth promotion, pathogen control, etc as stated above, these two compounds (amino acids and carbohydrates) in root exudates were selected which were measured by performing quantitative assay. In present study which was performed at Department of Biosciences in Saurashtra University at Rajkot (Gujarat) India, *Suaeda nudiflora* and *Cassia auriculata* were selected as experimental plants. *S. nudiflora*, a halophytic species, grows wild in the highly saline dry and extreme high tidal belt along the seacoast mainly in Gujarat. As seeds contain approx. 30-35% oil, plant has the potential as a future oil seed crop and is highly suitable for producing high protein biomass in saline soils due to C4 photosynthesis. It is occasionally used as fodder for camels and goats (Ramchandran K. *et al*. 1986) [42]. *C. auriculata* is known for its various medicinal properties like its bark is used as an astringent, leaves and fruits are anthelminthic, seeds used to treat in eye troubles and root employed in skin diseases (Siva, R., *et al.* 2005) [51], it is also used for the treatment of ulcers, leprosy and liver disease (Kumar, R.S., *et al.* 2002) [29], it's antidiabetic hypolipidemic (Umadevi, P., *et al.* 2006) and antioxidant (Kumaran, A., *et al.* 2007) [30] and hepato protective (Kumar, R.S., *et al.* 2003) [28] effect of have been reported, it's flower and leaf extract shown to have antipyretic activity (Vedavathy, S. *et al.* 1991) [61]. In present work, amino acids and carbohydrates in the root exudates of *Suaeda nudiflora* and *Cassia auriculata* were quantitative estimated. Protein/amino acids estimation were done by the methods Ninhydrin and Bradford's colorimetric assay while carbohydrate estimation was done by the methods Anthrone and Phenol-sulfuric Acid colorimetric assay.

2. Methodology

In present study amino acid estimation in *S. nudiflora* and *C. auriculata* root exudates composition was done by the Ninhydrin colorimetric assay (Spies, 2007) [53] and Bradford colorimetric assay (Bacilio Jimenez *et al*., 2003) [3] while the carbohydrate estimation in *S. nudiflora* and *C. auriculata* root exudates composition was done by Anthrone colorimetric assay (Brink *et al*., 1960; Aulakh *et al*., 2001) [7, 2] and Phenol - Sulfuric Acid colorimetric assay (Ashwell, 1966; Dubois *et al*., 1956) [1, 15].

2.1 Method adopted for growing plants in soil water culture for root exudates collection

Plants select those microbes contributing most to their fitness by releasing organic compounds through exudates creating a very selective environment where diversity is low (Saharan and Nehra, 2011) [46]. Seeds of *S. nudiflora* and *C. auriculata* were surface sterilized with freshly prepared 0.1% $HgCl_2$ solution for 2 minutes, washed with sterile distilled water, surface sterilized seeds of similar shape and size were inoculated on moistened filter paper in sterile petri plates and incubated for 72 h and seeds with no contamination were germinated in sterilized, washed soil. On the tenth day following sowing, the seedlings were carefully lifted after flooding the pots with distilled water. Roots were thoroughly washed free of all adhering sand particles with sterile water. Seedlings were transferred to 100ml flasks with cotton wool providing necessary aeration of the liquid substrate. The flask containing 3g of soil and 30ml of distilled water were autoclaved at 20 lbs in pressure for 30 minutes and cooled prior to transferring of seedling under aseptic conditions. The flasks were covered with thick brown-paper wrappings to cut out the effect of light on root growth. The plants were maintained for 25 days in the laboratory. The water level was maintained by addition of sterile distilled water on alternate days under aseptic conditions (Sulochana, 1962) [55].

2.2 Sampling of root exudates: After 25 days growth in the laboratory, the plants were carefully removed after washing the roots with distilled water (Sulochana, 1962) [55]. The root exudates of contamination free flasks were pooled, from which solution was filtered in order to remove root sheathing, root-border-like cells, rootlets, etc (Chaparro *et al*., 2013) [12] through filter paper and this filtrate was then used for quantitative assays of sugars (carbohydrate) and protein (amino acids).

2.3 Protein/amino acids estimation methods

2.3.1 Ninhydrin Colorimetric assay: Ninhydrin reacts with a free alpha-amino group, NH_2-C-COOH. This group is contained in all amino acids, peptides, or proteins. Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins. Different aliquots/concentration of standard (0.1/10, 0.2/20, ….0.8/80-ml/µg) and unknown sample (0.5ml) were taken. Volume was made to 1ml with D/W in all the tubes. 1ml of Ninhydrin solution was added in each tube and was mixed well. Tubes were kept in boiling water bath for 20 minutes. 5ml of diluent solution was added in each tube. Tubes were kept at room temperature for 15 minutes. And then OD was taken at 570 nm. Graph was plotted showing unknown concentration of amino acids/protein of experimental plants.

2.3.2 Bradford's Colorimetric assay

The Bradford assay is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response than the assays. Different aliquots/concentration of standard (0.2/20, 0.4/40, … . . 1.0/ 100 - ml/µg) and unknown sample (0.5ml) were taken. Volume was made to 1ml with D/W in all the tubes. 2ml of Bradfords' reagent was added in each tube and was mixed well. And then OD was taken at 595 nm. Graph was plotted showing unknown concentration of amino acids/protein of experimental plants.

2.4 Carbohydrate estimation methods

2.4.1 Anthrone Colorimetric assay: The Anthrone method is an example of a colorimetric method of determining the concentration of the total sugars in a sample. Different aliquots/concentration of standard (0.2/20, 0.4/40,…..1.0/100 - ml/µg) and unknown sample (0.5ml) were taken. Volume was made to 1ml with D/W in all the tubes. 4ml of Anthrone reagent was added in each tube and was mixed well. Tubes were incubated in boiling water bath for 15 minutes and then tubes were allowed to cool at room temperature. And then OD was taken at 620 nm. Graph was plotted showing unknown concentration of carbohydrate of experimental plants.

2.4.2 Phenol-Sulfuric Acid method: The Phenol - Sulfuric Acid method is an example of a colorimetric method that is widely used to determine the total concentration of carbohydrates. Different aliquots/concentration of standard (40/40, 80/80, …..,200/200 - µl/µg) and unknown sample (200µl) were taken. Volume was made to 200µl with D/W in all the tubes. 0.2ml 5% phenol and 1ml concentrated sulfuric acid was added in each tube. Tubes were kept at room temperature for 10 minutes mixed well & placed in water bath at 25-30^{0}C for 20 minutes. And then OD was taken at 490 nm. Graph was plotted showing unknown concentration of carbohydrate of experimental plants.

3. Result & discussion

Figure 1 shows the graph obtained by performing Ninhydrin colorimetric assay which shows the unknown concentration of amino acids/protein of experimental plants which was obtained from the standards taken in the assay and it was found that *C. auriculata* was showing better result than *S. nudiflora*. Figure 2 shows the graph obtained by performing Bradford's colorimetric assay which shows the unknown concentration of amino acids/protein of experimental plants which was obtained from the standards taken in the assay and it was found that *C. auriculata* was showing better result than *S. nudiflora*. Figure 3 shows the graph obtained by performing Anthrone colorimetric assay which shows the unknown concentration of carbohydrates of experimental plants which was obtained from the standards taken in the assay and it was found that *S. nudiflora* was showing better result than *C. auriculata*. Figure 4 shows the graph obtained by performing Phenol sulfuric acid colorimetric assay which shows the unknown concentration of carbohydrates of experimental plants which was obtained from the standards taken in the assay and it was found that *C. auriculata* was showing better result than *S. nudiflora*.

In root exudates of plants low molecular weight C compounds are present, including sugars, OAs and amino acids, are readily assimilated by microorganisms and may regulate the microbial community structure in the rhizosphere (Bais *et al*. 2006; Weisskopf *et al*. 2008) [5, 63], it could be expected that root exudates are important in plants and microorganisms interactions and may promote mutualistic associations between them (Bais *et al*. 2006; Koo *et al*. 2005; Lynch and Whipps 1990) [5, 27, 34]. Carbohydrates (arabinose, glucose, fructose, galactose, maltose, Raffinose, Rhamnose, ribose, sucrose, xylose) plays role in lubrication, protection of plants against toxin, microbial growth stimulation (Shengjing, 2009) [49]. Amino acids and amides (all 20 Proteinogenic amino acids, Aminobutyric acid, Homoserine, cystathionine, Mugineic acid phytosiderophores) inhibit nematodes and root growth of different plant species, microbial growth stimulation, chemoattractants, Osmoprotectants, iron scavengers (Shengjing, 2009) [49]. Several studies have shown that plant root-secreted phytochemicals mediate plant–microbe interactions in the soil. For example, the increased secretion of Chlorogenic acid and Caffeic acid and the decreased secretion of Cinnamic acid by grafted root watermelon improved its resistance to *Fusarium oxysporum f.* sp. niveum (Ling *et al*. 2013) [31]. Canavanine, secreted from the seed coat or roots of leguminous plants, acts as an antimicrobial for many rhizosphere bacteria but not rhizobia, suggesting that the host plant secretes this compound for selection of the beneficial microbes (Cai *et al*. 2009) [10].

Similarly, symbiotic associations between non-legumes and mycorrhizal fungi are mediated by root-secreted compounds, such as strigolactone 5-deoxystrigol (Yoneyama *et al*. 2008) [68], sugars (Fang and St. Leger 2010) [17], and carbohydrates (Kiers *et al*. 2011) [23]. In addition to these symbiotic interactions, root exudates are involved in the initiation of plant–PGPR interactions. PGPR are able to help plants through a variety of direct and indirect mechanisms. Plant roots are likely to attract PGPR through the release of cues (root exudates) in which carbohydrates and amino acids predominantly act as chemo attractants. Recent studies have shown that arabinogalactan

proteins (AGPs), which belong to the hydroxyproline-rich glycoprotein super family of plant cell wall proteins, play key roles in various interactions between plant roots and rhizospheric microbes in the rhizosphere (Nguema-Ona *et al*. 2013) [39]. Plant root tips release living root border cells, borderlike cells, and mucilage into the rhizosphere, which contains large amounts of AGPs (Cannesan *et al*. 2012; Hawes *et al*. 1998; Vicre *et al*. 2005) [11, 21, 62].

Although plant roots secrete AGPs abundantly into the rhizosphere, the role of AGPs in rhizospheric interactions has not been well studied. Recent studies have shown that AGPs are essential for plant– microbe interactions in the rhizosphere. For instance, AGPs are able to attract beneficial microbes (bacteria and fungi) and repel plant root pathogens (Cannesan *et al*. 2012; Gaspar *et al*. 2004; Vicre *et al*. 2005; Xie *et al*. 2012) [11, 18, 62, 66]. AGPs secreted by Arabidopsis root cap cells and border-like cells affect the colonization of *Rhizobium* sp., suggesting AGPs play important roles in recognition and attachment of rhizobia to the plant root surface (Vicre *et al*. 2005) [62]. In a recent study, a plant Arabinogalactan-like glycoprotein was found to be essential for the growth of bacteria on the roots of both legumes and non-legumes and was shown to promote the polar surface attachment by *Rhizobium leguminosarum* (Xie *et al*. 2012) [66]. The mechanisms by which AGPs influence the establishment and colonization of beneficial microbes to plant roots, how they shape the configuration of the microbial community, and other important functions of AGPs in the rhizosphere remain elusive. Hydroxyproline is a Ring-hydroxylated analog of Proline that is found abundantly in plant cell wall proteins (Cassab, 1998) [13]. Homoserine in pea extract comprises 79% of the total free amino acids and was found to be an inducer of a gene necessary for pathogenesis of the fungus *Nectria haematococca* in pea (Yang *et al*., 2005) [67]. So evidences are there that carbohydrate as well as amino acids in the root exudates of plant plays many inevitable roles throughout the life span of plant.

In present study result shows the presence of amino acids and carbohydrate in the root exudates of the *S. nudiflora* and *C. auriculata* which may act as an important nutrient source for plant growth promoting microbes (endophytes and rhizobacteria), attracts them to participate in the colonization process by inhibiting other pathogens and not only promotes plant growth but also prevent plant from pathogenic diseases (Bacilio-Jimenez *et al*., 2003) [3].

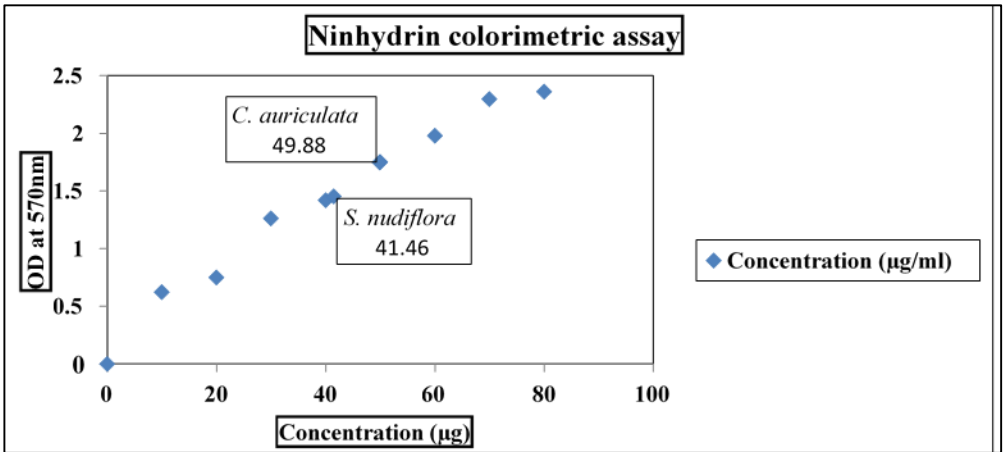

Fig 1: Graph obtained by performing Ninhydrin Colorimetric assay

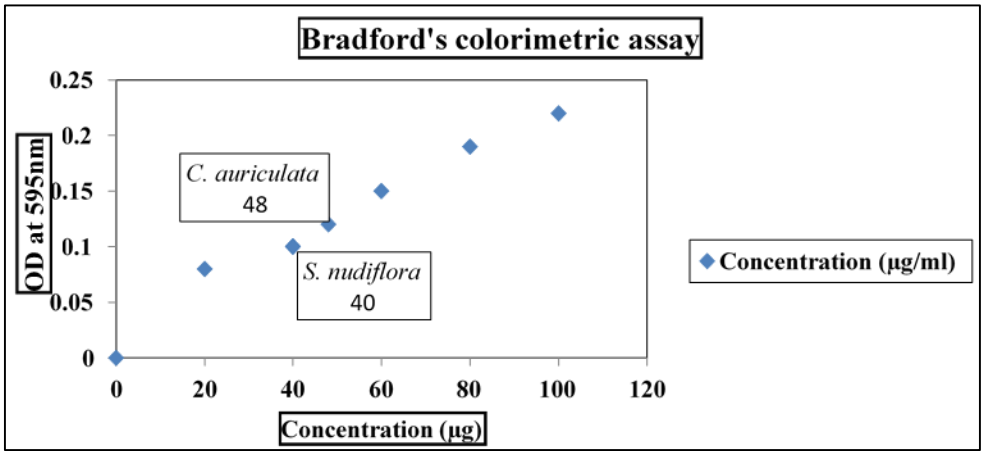

Fig 2: Graph obtained by performing Bradford's Colorimetric assay

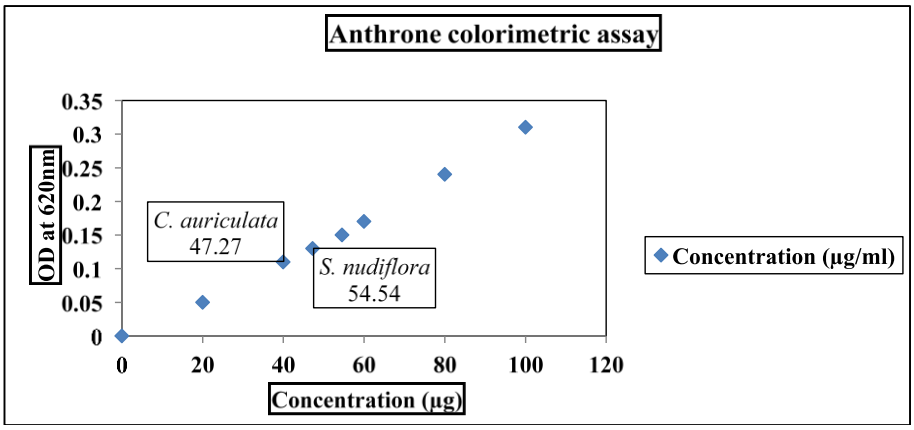

Fig 3: Graph obtained by performing Anthrone Colorimetric assay

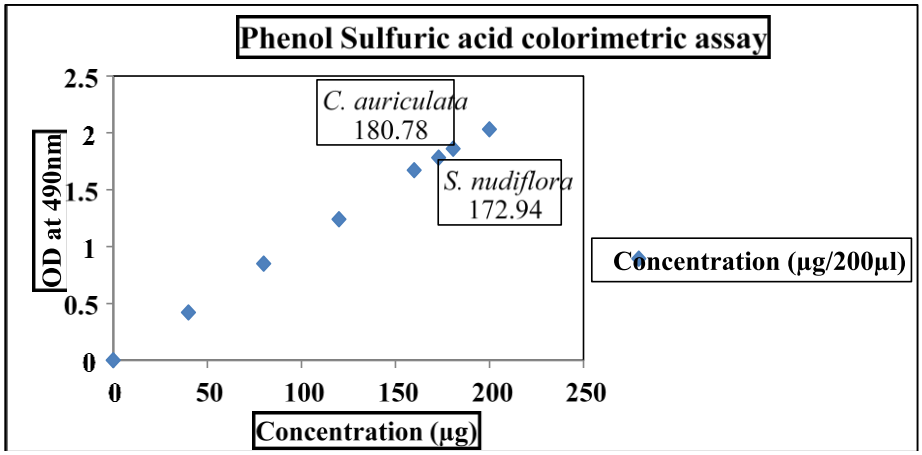

Fig 4: Graph obtained by performing Phenol-sulfuric acid assay

4. Conclusion

In the rhizosphere, root exudates plays an important role as attracting signal molecules to attract the plant growth promoting microbes (endophytes and rhizobacteria) which performs important plant growth promoting functions (biocontrol, hormone production and bioremediation). It can be concluded from the present study that, as experimental plants showed the presence carbohydrate and amino acids in root exudates, it will help in the promotion of plant growth, immunity and increase the resistance to arid and saline conditions which is not only of economic importance but also counted as contribution to the agriculture field.

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