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Isolation and Characterization of *Agrobacterium rhizogenes* from the Root Nodules of Some Leguminous Plants

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Abstract: A total of 57 colonies of *Agrobacterium* species was isolated from the root nodules of five different leguminous plant namely *Pisum sativum, Sesbania rostrata, Vigna mungo, V. radita* and *V. ungiculata* by using yeast extract mannitol agar (YEMA). All the isolated colonies were belonged to *Agrobacterium* species. The isolated bacteria were characterized and identified as *Agrobacterium rhizogenes* based on the morphological, biochemical, cultural and pathogenicity tests. Biochemical characteristics such as exo-polysaccharide, glycogen, total protein, total free amino acids and total lipids were estimated from the agrobacterial isolates. The agrobacteria of different leguminous plants showed lot of variations in their biochemical constituents.

Key words: Leguminous plants · *Agrobacterium rhizogenes* · Characterization · Biochemical constituents

INTRODUCTION

Bacteria within the genera *Agrobacterium* and *Rhizobium* have the unique capacity to induce prolific root formation, nitrogen fixing root nodules and autonomous crown-gall tumors on many higher plants including most dicots, some monocots and some gymnosperms [1]. *Agrobacterium* is a Gram negative, aerobic soil borne bacteria has worldwide distribution [2]. *Agrobacterium* spp. are commonly known as bacteria that infect dicotyledonous plant from over 90 different plant families including economically important fruit and nut crops, grapes ornamental and landscape plants.

The genus *Agrobacterium* belonged to the family Rhizobiaceae [3] which has been included in the alpha-2 subclass of *Proteobacteria* on the basis of ribosomal characteristics [4]. The cells are normally rot shaped (0.6-1μm by 1.5-3.0 μm), occur singly or in pairs, non-spore formers and are motile by one to six peritrichous flagella. Considerable extracellular polysaccharide slime is usually produced during growth on carbohydrate containing media.

Agrobacterium rhizogenes strain K84 (formerly called *A. radiobacter*) is used worldwide as a commercial agent for the biocontrol of crown gall disease caused by tumerigenic *Agrobacterium* strains [5]. Studies on the molecular characteristics and taxonomy of *Agrobacterium* and *Rhizobium* are plenty [6 - 9] but studies on the isolation and characterization of *Agrobacterium* from root nodules of legumes, especially pulses are meager or none. By keeping all these in mind, the present investigation was carried out with the isolation and characterization of *Agrobactierum* from the nodules of leguminous plant hosts such as *Pisum sativum, Sesbania rostrata, Vigna mungo, V. radiata* and *V. ungiculata* and estimation of biochemical constituents from the bacterial strains.

MATERIALS AND METHODS

Sample Collection: In the present study, the legumes such as *Pisum sativum, Sesbania rostrata, Vigna mungo, V. radiata* and *V. ungiculata* were collected along with root nodules from Tamil Nadu Agricultural University (TNAU), Kattuthottam, Thanjavur and also in and around Thanjavur district, Tamilnadu.

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Isolation of Agrobacterium: The nodules were detached carefully and sterilized thoroughly as per the standard procedure of Sharma *et al.* [10]. The nodules were kept immersed in 0.1% acidified mercuric chloride solution for 5 min. and washed repeatedly with sterile distilled water. Then they were immersed in 70% ethyl alcohol. This treatment was followed by repeated washing with sterile distilled water. These sterilized root nodules were crushed simply with pestle and mortar and extracted with sterile distilled water. *Agrobacterium* isolates were isolated by using serial dilution and pour plate techniques. The root nodule extract was serially diluted up to 10^{-9} with sterile distilled water and 1 ml of diluted sample was inoculated into sterile Petri plates and poured with the sterilized YEMA medium [11], plates were incubated at 28°C for 2 to 3 days. This medium allowed both *Agrobacterium* and *Rhizobium* to grow and develop into colonies. After incubation, the bacterial colonies were purified by streak plate technique on D1 medium. Pure agrobacterial cultures were maintained in both *Agrobacterium* mannitol medium [g/l: tryptone 5; mannitol 5; yeast extract 2.5; L-glutamic acid 1; KH_2PO_4. 25; NaCl 0.1; $MgSO_4.7H_2O$ 0.1; biotin 10 µl (0.1 mg/ml stock); pH 7.0; agar 15] and *Agrobacterium* D_1 medium (Sigma) [g/l: MS salts 4.3; glucose 3; B5 vitamins (1X); zeatin 1mg/l; pH 5.8; agar 0.8].

Characterization of Agrobacterium Isolates: The *Agrobacterium* isolates was characterized based on the cultural, biochemical and physiological characteristics such as Congo red test [12], Hofer's alkaline broth test [13]. Growth in glucose peptone agar [14], reaction of litmus milk [11], staining of poly â-hydroxybutyrate (PHB) [15] and *Agrobacterium* specific tests such as growth on potato dextrose agar (PDA), 3-ketolactose test, sodium chloride tolerance test, growth and pigmentation in ferric ammonium citrate broth test and citrate utilization test were also carried out. Also, all the *Agrobacterium* isolates was confirmed by the pathogenicity test i.e., hairy root formation. Further, biochemical studies such as estimation of exo-polysaccharides, glycogen, total protein content of the *Agrobacterium* isolates from the five different host plants was carried out by the standard methods of Lowry *et al*. [16] and free amino acids of the isolates was also been estimated by the standard procedures of Jayaraman [17].

RESULTS AND DISCUSSION

In the present study, totally 57 agrobacterial colonies were isolated from the nodules of leguminous plants namely *Pisum sativum, Sesbania rostrata, Vigna mungo, V. radita* and *V. ungiculata.* In YEMA medium, the agrobacteria absorbed Congo red, but the rhizobia were not and the colony morphology of agrobacteria was similar to that of rhizobia. The isolates showed well pronounced growth in glucose peptone agar and Hofer's alkaline broth at pH 11. Yellow colouration was found in lactose agar with Benedict's reagent. All these tests have already been conducted and reported by Hofer [13] and Klecz-Kowska *et al.* [18] for the characterization of agrobacterial isolates. They confirmed that those organisms which have the above said characteristics are used to identification of *Agrobacterium*. This confirmed that the findings of the present study have been isolated *Agrobacterium* species from the root nodules of leguminous plants but not *Rhizobium*.

The confirmation of *Agrobacterium* was made by the specific tests *viz*, growth on PDA medium, 3-ketolactase test, growth and pigmentation in ferric ammonium citrate containing media, sodium chloride tolerance test and citrate utilization test were conducted for all the strains. On PDA medium, the isolates showed well pronounced growth, similarly higher concentration of sodium chloride did not affect the growth of isolates and they utilized citrate. Similar types of tests have already been conducted by Moore *et al.* [19]. Gaur *et al.* [20] distinguished *Agrobacterium* from *Rhizobium* by 3-ketolactose test, whereas the *Agrobacterium* produced yellow ring of precipitate of CuO_2 around the colonies of the bacterium when plates were flooded with Benedict's reagent. Clark [21] also applied the above test to distinguish *Agrobacterium rhizogenes* from *A. tumifaciens, A. rubi, A. pseudosugar* and *Rhizobium trifoli*. He found that *A. rhizogenes* was positive whereas all the other species were negative. In the present study all the isolates were showed positive results for 3-ketolactose test. From the above specific tests it was confirmed that the isolates from different legumes are *Agrobacterium* and not *Rhizobium*.

To identify the species of *Agrobacterium*, pathogenicity test was conducted by seed inoculation technique. All the agrobacterial species isolated from different leguminous plants showed profusely branched secondary roots. An interesting point was noted that when *Agrobacterium* alone was inoculated into the host species, there was the formation of proliferate and multi-branched secondary roots from all species tested. On the other hand, when *Agrobacterium* along with *Rhizobium* (of the host species) were inoculated, there were not much branched secondary roots in any of

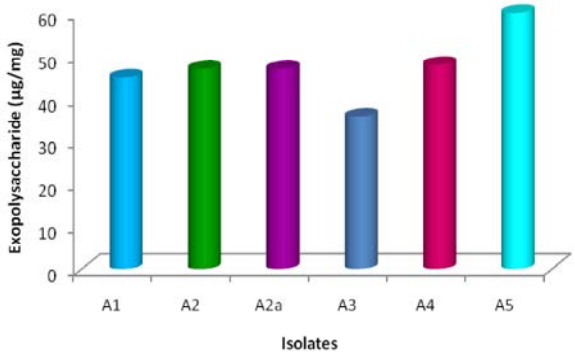

Fig. 1: Exo-polysaccharide content of *Agrobacterium rhizogenes* isolated from the nodules of different hosts

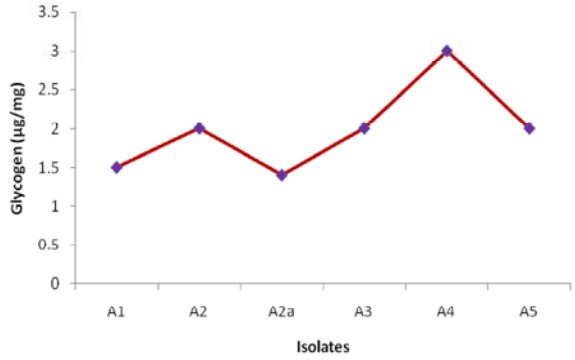

Fig. 2: Glycogen content of *Agrobacterium rhizogenes* isolated from the nodules of different hosts

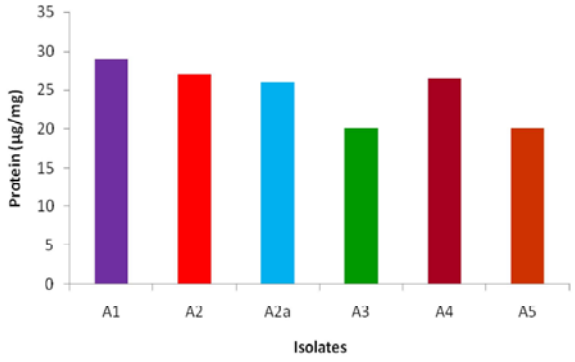

Fig. 3: Protein content of *Agrobacterium rhizogenes* isolated from the nodules of different hosts

the host species tested, instead the formation of nodules was observed. Similarly when *Rhizobium* alone was inoculated, all the host species showed the production of well developed nodules. From the above results it is concluded that the agrobacteria isolated from the nodules of different legumes were *A. rhizogenes* moreover when *Rhizobium* present along with *Agrobacterium*, it could inhibit the formation of hairy roots in the host species,

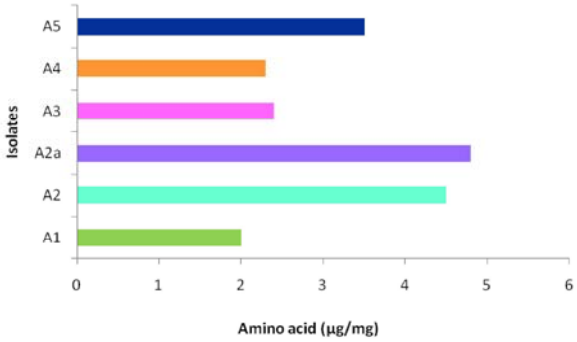

Fig. 4. Free amino acid content of *Agrobacterium rhizogenes* isolated from the nodules of different hosts

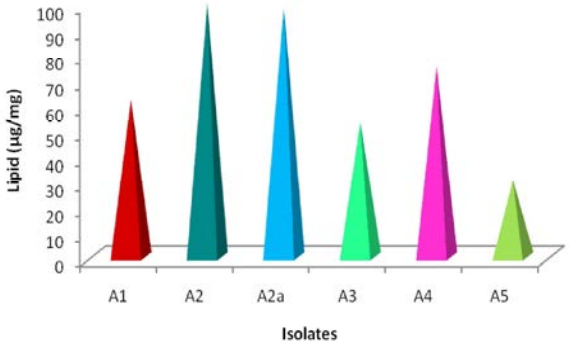

Fig. 5: Lipid content of *Agrobacterium rhizogenes* isolated from the nodules of different hosts

but *Agrobacterium* would not affect the formation of nodules by *Rhizobium*. Similarly, hairy root production test has already been reported by several workers [22-25].

In order to compare the stains of *A. rhizogenes* isolated from different leguminous plants, biochemical studies such as estimation of exo-polysaccharide, glycogen, total proteins, free amino acids and total lipids were carried out. The strains of agrobacteria isolated from the nodules of different host plant species showed remarkable differences in their biochemical constituents. The maximum (60 µg/mg) content of exo-polysaccharide was observed in *Agrobacterium* of *V. ungiculata* and minimum (36 µg/mg) was observed in *Agrobacterium* of *V. mungo,* whereas the other species were did not show much variation in exo-polysaccharide contents (Fig. 1). On the other hand, glycogen content was maximum as 3 µg/mg (Fig. 2) in *Agrobacterium* of *V. radiata* and minimum as 1.4 µg/mg was observed in *S. rostrata* (stem) isolates. The protein content was high as 29 µg/mg in *Agrobacterium* of *Pisum sativum* and low as 20 µg/mg in *Agrobacterium* isolates of both *V. mungo* and *V. ungiculata* (Fig. 3). Contrary to this, the free amino

acids content was maximum (4.8 µg/mg) in the agrobacterial isolates of *S. rostrata* (stem) followed by the agrobacterial isolates of *S. rostrata* (root), *V. ungiculata, V. mungo, V. radiata* and *Pisum sativum* (Fig. 4). Similarly, the maximum (100 µg/mg) content of total lipid was (Fig. 5) recorded in *Agrobacterium* from root nodules of *S. rostrata* followed by *S. rostrata* (stem) *V. radiata*, *P. sativum*, *V. mungo* and *V. ungiculata*.

In the present study, from the above discussion it is clear that the biochemical studies showed lot of variations within the same species of *Agrobacterium* from different leguminous plants and it may be concluded that the isolates of *A. rhizogenes* be treated as separate strains.

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