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Isolation and phenotypic characterization of *Alysicarpus* from arid region

CHANDRAVEER AND MANISH CHITTORA

In this research work, nodulation in *Alysicarpus vaginalis* were studied and also root nodulating bacteria were phenotypically characterized. Nodules were found associated with tap roots as well as on lateral roots. The number of nodules/plants varied according to the sampling site characterized by type of soil, soil moisture and nutrients. In *Alysicarpus vaginalis*, number of nodules (>100) in well water conditions was found. In field conditions too the number of nodules/ plants was high in *Alysicarpus vaginalis*. Rhizobia have been isolated from root nodules of *Alysicarpus vaginalis* a native legumes species growing in arid and semi arid regions of Western Rajasthan. Rhizobia isolates were Gram negative, small rods. Typical rhizobial colonies (raised, lens shaped, white translucent, watery, some time gummy, mucous etc.) were obtained, purified and used for phenotypic characterization. Rhizobial isolates from *Alysicarpus vaginalis* observed to be slow growing hence, low acid production was observed. It possessed sensitivity toward test antibiotics used in this study.

Key Words : Nodulation, Legumes, Arid region, Bacterial colony, Slow growing rhizobia

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INTRODUCTION

Alysicarpus vaginalis is a summer annual legume long grown as a grazing and hay crop. The plant may grow to a height of three feet at maturity. While traditionally a forage and hay crop, alyce clover is also used as a cover/green manure crop and provides the advantages of good nitrogen and biomass production. Broadcast seeding rates are 15-20 Ibs/ acre, and seed should be covered one-fourth to one-half inch deep. Seed can be planted by drilling or broadcasting and cult packing into a well-prepared seedbed. Planting may begin in April and extend through early June when adequate moisture is available for good germination. Alyce clover grows best in well-drained soils with moderate to good fertility levels. The crop does not tolerate acid soils well, and pH should be in the range of 5.5 to 6.5 for best growth. Few disease problems or insect pests affect crop establishment and subsequent growth. However, the plant is highly susceptible to root-knot and sting nematodes and sites with a history of nematode problems should be avoided. In many cases, susceptibility to nematodes renders this crop less than desirable as a cover crop. Alyce clover will reseed when allowed to grow to maturity and this should be considered when determining crop termination to prevent creating a potential weed problem in succeeding crops.

In the present investigation *Rhizobium* sp. associated with legume *Alysicarpus vaginalis* were isolated and purified. Biochemical characterization and antibiotic sensitivity test were also performed for confirmation. They were further studied for morphology and developmental pattern of nodules and their internal structure. The Rhizobic species associated with *Alysicarpus vaginalis* are studied. The Rhizobic species were isolated and cultivated on nutrient media. Biotyping was done on the basis of biochemical and morphological characteristics.

The *Alysicarpus vaginalis i.e.* native nodulating legume

MEMBERS OF RESEARCH FORUM

Author for correspondence :

CHANDRAVEER, Department of Dairy and Food Microbiology, College of Dairy and Food Science Technology, Maharana Pratap University of Agriculture and Technology, UDAIPUR (RAJASTHAN) INDIA
Email: chandraveercharan@gmail.com

Associate Authors' :

MANISH CHITTORA, Department of Dairy and Food Microbiology, College of Dairy and Food Science Technology, Maharana Pratap University of Agriculture and Technology, UDAIPUR (RAJASTHAN) INDIA
Email: mnshchittora@yahoo.co.in

of the arid region of Indian Thar desert will make base for more elaborative studies on BNF in this region. Wild legumes are source of genetically diverse Rhizobia that may have ability to survive harsh conditions and will prove useful in agriculture (Zahran, 2001). The information on nodulating native *Alysicarpus vaginalis* and associated Rhizobia of arid region will help in making status report of native Rhizobia of the stress ecosystem. This will ultimately benefit in formulating consortium of agriculturally important microorganisms in India. The wide range of tolerance to pH, NaCl, antibiotic resistance and production of high EPS by the desert Rhizobia are useful traits that can be exploited in the future. There is need of exploring more sites having diverse ecological niche for isolating Rhizobia from extreme environment of the Indian Thar desert.

Isolation of effective Rhizobia from wild legumes like *Alysicarpus vaginalis* can be very effective to improve quality of arid land. *Alysicarpus vaginalis* had already known for rhizobic association. Bhattacharyya and Patti (2000) isolated and identified a *Rhizobium* sp. from root nodules of *Alysicarpus vaginalis* and also described growth behaviour and indole acetic acid (IAA) production by a Rhizobium isolates. Leigh and Coplin (1992) studied the tumor like structure formed by Rhizobium species on roots of *Alysicarpus vaginalis*.

Members of papilionoideae found in arid zone:

Papilionoideae is the largest of the legume sub families and is the one containing the highest proportion of nodulated species. All known types of nodules are found in this sub family. The nodulated genera of various tribe found in arid and semi arid regions are *Abrus* from Abreae, Cicer from Cicereae, *Crotalaria* from Crotalarieae, *Aeschynomene* and *Dalbergia* from Dalbergieae.

General characteristics of *Alysicarpus vaginalis*:

Common names:

Alyce clover, buffalo clover, one-leaf clover, trebol Alicia.

Morphological description:

Low growing annual or short-lived perennial, extremely variable in habit, leaf shape and flower colour. Stems numerous, 10-100 cm long, emanating from the rootstock; variable in hairiness, moderately branched and leafy; with single simple oval-shaped leaves on a short petiole 10 mm long with prominent pointed stipules. Leaves 5.65 x 3.25 mm, but generally 10 x 20 mm. Flowers, 6 mm long, reddish yellow or pale purple, borne in racemes up to 13 cm long and comprised of 6-12 flowers each. The seeds, dark red, oval or oblong, 1-1.5 mm long. Seed weight is approximately 625 seeds/g for cultivar 'FL5' and higher for cultivar 'FL3'. Ungrazed plants can be more erect , growing to 1 m in height in dense swards.

Uses/applications:

A. vaginalis is a useful component of native pastures, especially under heavier grazing. It is cultivated for pasture, hay (in the United States) and forage. It is also used for soil improvement and conservation and provides effective erosion control on newly established terraces.

Ecology:

Soil requirements:

A. vaginalis grows on a wide range of soil types from coralline sands to moderately acid clays. It has moderate fertility requirements and will respond to P and K fertilizers when growing on infertile soils. Its susceptibility to nematodes can limit productive growth to heavier soils, including black clays. Low salinity tolerance.

Moisture:

It will grow in the humid, sub-humid tropical and subtropical lowlands, under rainfall from 900-2,000 mm. Under wetter conditions, it behaves as a perennial. It can stand dry seasons of up to 6 months but may behave as an annual in more arid regions. It does not like waterlogged conditions but can tolerate short-term flooding.

Temperature:

Broad adaptation to temperature, from warm temperate regions to the tropics, and from sea level to 1,400 m as in many tropical areas. Leaves killed by light to moderate frosts. *A. vaginalis* is killed by heavy frosts but generally regenerates strongly from seed in the following spring/summer.

Light:

Alyce clover grows well under moderate shade and is more vigorous under the canopy of shrubs rather than in the open. Similar shade tolerance to Desmodium heterocarpon subsp. ovalifolium in a greenhouse study in Malaysia.

Reproductive development:

A short-day plant producing relatively high seed yields in the first season of growth. Seed requires an after-ripening period of approximately 16 weeks to overcome physiological dormancy. Physical dormancy must also be overcome by abrasive scarification to break the seed coat.

Defoliation:

Alyce clover is very tolerant of continuous, heavy grazing and regular mowing. Under grazing conditions, single plants change from an erect form of growth to a small, flattened rosette. It is likely that tall erect growth cut at a

low level will not recover quickly due to the absence of growing points.

Fire:

Fire is uncommon in the heavily grazed swards that favour Alyce clover. A perennial accession of Alyce clover (IRFL 3240) persisted and spread following burning in Florida due to its deep, well-developed crown. Very hot fires may kill the plant but the species will recover from seed.

Establishment:

Commercial seed of *Alysicarpus vaginalis* is seldom available. It has been planted as a hay crop in Mississippi and Florida with seed rates of 10-15 kg/ha, sown into disced strips. Seed for immediate germination should be scarified but does not require specific inoculants. No longer commonly planted as an improved pasture or hay crop.

Fertilizer:

Alyce clover responds well to P, K and S on deficient soils in both native pastures and sown stands.

Compatibility (with other species):

Alyce clover declines under intense competition from vigorous grasses, but combines well with native grasses controlled by heavy grazing. It can combine well with creeping, sward -forming grasses under grazing and frequent cutting as in lawns.

Companion species:

Grasses: *Stenotaphrum secundatum*, *Bothriochloa pertusa*, *Dichanthium caricosum*

Legumes:*Desmodium heterophyllum*, *Atylosia scarabaeoides* (as a useful component of naturalised pastures on basaltic slopes at Sigatoka, Fiji).

Pests and diseases:

Alysicarpus vaginalis is very susceptible to root-knot nematodes (*Meloidogyne* spp.). As these are more severe on light soils, the legume often grows best on heavier soils. Leaves are often severely affected by leaf-mining caterpillars.

Ability to spread:

Alyce clover spreads naturally under grazing probably aided by spread of seed in dung. It becomes more prevalent under grazing.

Weed potential

Common weed of lawns and golf fairways where *A. vaginalis* persists under regular mowing.

Nutritive value:

Crude protein content 16–18 per cent and *in vitro* dry matter digestibility of 67–73 per cent.

Palatability/acceptability:

Well eaten by cattle and horses. Similar palatability to lucerne (*Medicago sativa*) and *Aechynomene americana* under grazing by sheep in a cafeteria trial.

Toxicity:

Reported not to cause bloat in cows, presumably due to the presence of condensed tannins in the forage.

METHODOLOGY

Field survey:

The survey was restricted mostly around Jodhpur specifically Osian, Pali, Jadan, Surpura, Mandore, Daijar, Kayalana Machia Safari Park, Chokha village, Kudi-Bhagtasani and New Campus, J.N.V. University. Beside this some collections were also made from Sikar-Fatehpur area. The survey was conducted during monsoon and post monsoon period (July–September) .

Collection of germplasm:

Valuable germplasm of *Alysicarpus vaginalis* was collected from field for further studies. Mature pods were collected and seeds were stored in small plastic container with mercury tablets and desiccant. Seeds were stored at low temperature.

Recording of climatic data:

Climatic data (Meteorological data) for Jodhpur and Jaisalmer was obtained from Meteorological department of Central Arid Zone Research Institute, Jodhpur. Rainfall data for other sampling site were also obtained from CAZRI, Jodhpur.

OBSERVATIONS AND ASSESSMENT

The experimental findings obtained from the present study have been discussed in following heads:

Nodule hunting : A survey of native nodulating legumes of arid and semi arid regions of W. Rajasthan:

A massive field survey of native nodulating legumes of arid and semi arid regions of W. Rajasthan were conducted during the monsoon (July-Aug.) and post monsoon periods for knowing their nodulation status in natural habitat. The surveys were conducted around Jodhpur including Mandore, Surpura village, Osian, Pali, Jadan, Chokha village and some part of the Sikar and Fatahpur. Study was performed on *Alysicarpus vaginalis* (Linn.) DC nodulation from the root nodules of *Alysicarpus vaginalis* DC. the symbiont was isolated and identified for the presence of Rhizobium species.

Arrangement of nodules on root:

Nodule of *Alysicarpus vaginalis* found in field (arid and hard soil) is difficult to trace intact and, therefore, to know their arrangement on root as well as their accurate number they were studied from plant growing in pots in rhizospheric soil. Plant was harvested from pots at pre-flowering, flowering and post-flowering stages. Nodules were distributed singly as well as in cluster on main and lateral roots in all investigated legume species. All nodules of *Alysicarpus vaginalis* were originated from root axils while in rest of the species they were originate far from root axils.

Development, morphology and other characteristics of nodules:

Nodules of all developmental stage were collected after flowering stage from an individual plant growing in pots. Plant shows nodules at various developmental stages. Young nodules were globose or papilla shape in almost all species but later on develop into various shape and size according to plant species. Various characteristics of nodules as described by Corby (1988), *viz.*, type of nodules, their shape, size, number, colour and arrangement on root are documented. Nodules of all species were mainly divided into two type *viz*., determinate and indeterminate and further classified into different categories as described by Corby (1981 and 1988). Nodules of *Alysicarpus vaginalis* were originated from root axils which are the typical characteristic of aeschynominoid nodule but there are true prominent lenticels present on surface and interstitial uninfected cells are present in infected zones of root nodule which are the characteristics of desmodioid type nodules hence, were assumed as hybrid of aeschynominoid and desmodioid type. Nodules were smooth in texture in *Alysicarpus vaginalis*. Periderm were also present on nodules of *Alysicarpus vaginalis*. Prominent ridge shape lenticels (openings for aeration) were present on radial arms of nodules of *Alysicarpus vaginalis*. Nodule size was found to be ranging from 0.5 mm to 1.5 cm.

Presence of leghemoglobin:

The middle half sections of nodules of *Alysicarpus vaginalis* showed red/pink or orange colour inside the nodule. It was due to the presence of leghemoglobin. The presence of leghemoglobin showed that these all nodules were active nodules and were actively fixing atmospheric nitrogen.

Microbiological studies on Rhizobia associated to native legumes:

Rhizobia associated with root nodules of *Alysicarpus vaginalis* were isolated. The isolates from root nodules were identified as rhizobial isolates on the basis of Gram staining, morphology, and colony characteristics on Congo-red YEMA media. Primarily these were classified into acid producing and alkali producing on the basis of reaction with BTB-YEMA, and reaction with litmus milk.

Phenotypic characterization of isolated Rhizobia:

Isolated Rhizobia were Gram negative and showing motility. Rhizobial colonies on CR-YEMA were showing no absorption of dye but however, after long time culturing some of the colonies absorbed red dye. The other phenotypic characteristics observed are litmus milk reaction, pH tolerance, NaCl tolerance, and intrinsic antibiotic resistant properties.

Colony characteristics:

Colony characteristics of Rhizobia isolated from *Alysicarpus vaginalis* were studied. Slow growing rhizobial colonies were isolated from *Alysicarpus vaginalis*. The colonies appearance were creamy white, circular, raised, lens shaped with entire margin.

Gram staining and morphology of isolated Rhizobia:

Rhizobia isolated were Gram negative and rod shaped. Rhizobia were observed to be not accurate rod shaped instead they appeared oblong, globular, and pear shaped. The bacteroids were also observed in slides prepared from crushed nodules. Bacteroids were of rod shape and were comparatively larger in size as compared to cultured Rhizobia.

Growth on congo-red (CR) YEMA :

Isolated bacterial colonies from *Alysicarpus vaginalis* did not absorb red dye and remained colour less. As described above bacterial colonies from this plant is slow growing hence unable to absorb dye at fast rate.

Growth on bromothymole blue (BTB)-YEMA medium:

Observation was taken as change in coloration which was due to formation of acid. Moderately growing Rhizobia isolated from *Alysicarpus vaginalis* produce low acid and change the BTB into light yellow or yellowish green.

Growth on peptone glucose agar (PGA) medium:

On PGA media low growth of Rhizobia was observed isolated from *Alysicarpus vaginalis*.

Motility of isolated Rhizobia:

Rhizobia isolated from *Alysicarpus vaginalis* showed motility in broth media.

Reaction on litmus milk:

Isolates from *Alysicarpus vaginalis* leads rennet curd formation with no serum and gas was on litmus milk media. Litmus was observed to be reduced into white colour.

Growth rate of isolated Rhizobia:

The generation time (GT) was observed to be 1-2 hrs for isolates from *Alysicarpus vaginalis*.

pH tolerance of isolated Rhizobia:

Slow growing Rhizobia were isolated from *Alysicarpus vaginalis*. They were able to grow on pH range of 4 to 11 (Table 1).

NaCl tolerance of isolated Rhizobia:

NaCl tolerance of Rhizobia were studied by using NaCl concentration of 0.25 to 9 (w/v). Rhizobia isolated from *Alysicarpus vaginalis* showed tolerance to NaCl up 1% (Table 2).

Antibiotic sensitivity of isolated Rhizobia:

Isolates from *Alyicarpus vaginalis* showed varied intrinsic antibiotic resistant depending upon the type of antibiotic used. The effect was dependent on amount of antibiotic as well as type of antibiotic (broad/narrow spectrum). The two antibiotic used were ceftriaxone 25 meg/ disc and cefotoxime 10 meg. Antibiotic sensitivity to antibiotics was measured in the form of zone of inhibition. The size of inhibition zone made by isolates with respective to particular antibiotic disc is documented in Table 3. The results also shows that all isolates clearly differed from one another.

In the present investigation *Alysicarpus vaginalis* growing in arid and semi-arid regions around Jodhpur (Mandore, Kayalana, Surpura, Daijar, Jhalamand, Mathania, Osian), Pali district (Sojat and Jadan) and Sikar (Fatehpur area) was evaluated for occurrence of nodulation. Root-nodulating bacteria were isolated and characterized from *Alysicarpus vaginalis* growing in arid and semi-arid regions. Phenotypic characteristics measured included growth rates in various media, colony morphology, and tolerances to salt and pH nodulation. This belongs to *Papilionoid* genera. The nodulation in some of the species of these above mentioned genera have been reported by other workers from India but not from the arid ecosystem of Indian Thar desert (Subramaniam and Babu, 1994; Jha *et al*., 1995).

The total number of nodules per plant and size of the nodules varied depending on soil moisture and characteristics of soil. In pots if sufficient soil moisture was maintained the number of nodules per plant exceed significantly. The maximum number of nodules/plant (>100) were found in *Alysicarpus*, grown in earthen pots and watered regularly to maintain good soil moisture. In case of number of root hairs of *Alysicarpus vaginalis*, numerous root hairs were observed. Soil pH has major impact on the nodulation process and has been extensively examined. Low pH had more severe impact on survival and multiplication of Rhizobia (Brockwell *et al*., 1991). Dhir (1977) also recorded alkaline pH (8.2 to 8.8) at various depths (0-15 cm to 90-135 cm).

Root nodules of *Alysicarpus vaginalis* showed typical characteristics of a legume nodule characterized by the central infection zone and a peripheral vascular supply (Pawlosky *et al*., 2003, Sprent, 2001). In present investigation Rhizobia were isolated from root nodules and phenotypically characterized. The rhizobial colonies were characterized as size and appearance, as described by Zahran (2001). Isolates from *Alysicarpus vaginalis* observed to be slow growing. As these isolates are slow growing hence, low acid producing Rhizobia isolated on BTB. *Alysicarpus vaginalis* also showed tolerance to alkaline pH and show tolerance up to 1% of NaCl. Similarly the Rhizobia isolated from arid regions showed the characteristics feature of production of high exopolysaccharides (EPS). The high production of EPS by fast growing Rhizobia is advantageous for successful root infection/rhizobial plant interaction such as symbiotic nodule development (Leigh and Coplin, 1992). Compelling evidences suggest that gene responsible for EPS biosynthesis is involved in the infection process and possible host range specificity (Phillip-Hollingsworth *et al*., 1989). The high EPS accumulation

Table 1: pH resistant test in Rhizobia isolated from *Alysicarpus vaginalis*

Sr. No.	Name of the host	pH										
		2	3	4	5	6	7	8	9	10	11	12
1.	*Alysicarpus vaginalis* (Linn.) DC.	-	-	+	+	+	+	+	+	+	+	-

Table 2: NaCl resistance in Rhizobia isolated from native legumes

Sr. No.	Name of the host	Percentage of salt (W/V)										
		0.25	0.5	0.75	1	2	3	4	5	6	7	8	9
1.	*Alysicarpus vaginalis* (Linn.) DC.	+	+	+	+	-	-						

Table 3: Antibiotic resistance in Rhizobia isolated from native legumes

Name of host	Antibiotic con. mcg/disc (Zone of inhibition in mm)							
	Ceftriaxone 25 mgc/disc				Ceftriaxone 10 mgc/disc			
	R1	R2	R3		R1	R2	R3	
Alysicarpus vaginalis	28	29	31	29.33	28	29	28	28.33

may have beneficial for maintaining moisture in arid regions and, therefore, helpful in nodulation in unfavorable conditions. The characterization of biopolymer of EPS produced by the desert Rhizobia may have other applications in biotechnological as well as nanotechonological approaches. In addition these isolates also showed sensitivity towards antibiotic employed in this study *i.e.* ceftriaxone and cefotoxime.

In conclusion, isolates from present study may be useful to increase the symbiotic nitrogen fixation in legumes used for forage and green manure. More biodiversity studies are wanted to screen nitrogen fixing organisms from different ecosystems, with emphasis on their biochemical characteristics. Continued efforts should be made to understand the complex association between legumes and their symbiotic partners, with an emphasis on their ecological role, co-evolution, selection of symbiotic partners, acquisition, soil fertility, evolution and transfer of nitrogen fixation (nif) or nodulation (nod) genes, and ultimately to employ efficient strains in the sustainable agricultural practices.

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