Tree and Forestry Science and Biotechnology ©2007 Global Science Books

Robinia pseudoacacia Linn.

Kamlesh Kanwar* • **Anju Bhardwaj** • **Raj Deepika** • **D. R. Sharma**

Department of Biotechnology, Dr Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, 173 230, India

Corresponding author: * **kanwarkamlesh@hotmail.com**

ABSTRACT

Robinia pseudoacacia L. is a nitrogen-fixing, leguminous, fast-growing and multipurpose agroforestry tree adapted to drought-affected and degraded sites. Recent interest in woody biomass as a source of fuel has fostered additional investigations into the culture of *R. pseudoacacia*. In trials of potential biomass species, it demonstrated superior growth rates and energy yields. However, because of its rapid growth, early flowering, adaptability to drought and degraded sites it is widely planted through out the world's temperate zone for multiple purposes. These advantages suggest the possibility of selecting variants for tree improvement. Improvement by conventional tree improvement programmes is cumbersome and time-consuming, while *in vitro* propagation techniques offer an alternative approach. This species has been regenerated *in vitro* by micropropagation, protoplast and cell suspension culture. These biotechnological tools can be used for mass propagation, induction of genetic variability, production of disease-free and stress-tolerant plants from isolated cells, protoplasts and tissues and introduction of transgenic trees following molecular characterization.

Keywords: cell suspension culture, *in vitro* nodulation, genetic transformation, micropropagation, molecular markers, protoplast culture

CONTENTS

INTRODUCTION

Robinia pseudoacacia Linn. is a papilionaceous multipurpose tree legume. It is native to the south-eastern USA, which has become neutralized in many countries worldwide (Harlow *et al*. 1979). It was first introduced in the mid hill zone of North Western Himalayan region of India (Troup 1921) between an altitudinal range of 1000 to 3000 m (Muttoo and Kango 1965). Black locust is one of the most widely planted broad leaf species in the world equally ranked with *Populus* and second to *Eucalyptus* in total hectares established (Kereszteski 1981).

Received: 6 March, 2007. Accepted: 23 August, 2007.

Invited Mini-Review

R. pseudoacacia is among the few leguminous nitrogen-fixing trees adapted to frost-prone areas. It is also adaptable to environmental extremes such as drought, air pollutants, and high light intensities (Hanover 1989) and is of special interest for intensive forest improvement. It is widely known for rapid growth, ecological plasticity, adaptability and good timber quality. It is remarkable for its ornamental value as it bears white, fragrant flowers and a deep, spreading root system which is capable of growing in a wide variety of soils but not on very sandy, very acidic or wet soils. It is an invaluable species for road side avenues (Muthoo and Kango 1965) and can be used as a landscaping tree.

The last few years have shown the biotechnology of woody angiosperms and gymnosperms to fruition since major advances in this recalcitrant group of species have taken place. Protocols for micropropagation (Kanwar *et al*. 1996), assessment of micropropagated plantlets (Kanwar *et al*. 2002; Kaushal and Kanwar 2003), protoplast culture (Bharadwaj 1999), cell suspension culture (Kanwar *et al*. 2007) and genetic transformation (Kanwar *et al*. 2003) of *R. pseudoacacia* have been developed. So, *in vitro* methods can be used to obtain true to type in a short span of time, irrespective of season and introduction of agronomically important genes by genetic transformation.

TAXONOMY

The currently accepted scientific name for black locust is *R. pseudoacacia*. There are no natural subspecies or forms, but many cultivars are available. Named varieties are as follows: *R. pseudoacacia* var. *pseudoacacia*; *R. pseudoacacia* var. *rectissima* (L.) Raber (Little *et al*. 1979; Kartesz *et al*. 1980; Huntley 1990).

Black locust hybridizes with Kelsey locust (*Robinia kelseyi*), New Mexico locust (*R. neomexicana*), clammy locust (*R. viscosa*), and bristly locust (*R. hispida*).

COMMON NAMES

Black locust, false acacia, yellow locust, white locust, green locust, post locust, shipmast locust and locust.

Fig. 1 *Robinia pseudoacacia* Linn.

BOTANY

Black locust is a medium-sized, native, deciduous tree. Mature height ranges from 40 to 60 feet (12-18 m), and 12 to 30 inches (30-76 cm) (**Fig. 1**). Black locust grows rapidly, reaching mature heights in 20 to 40 years. It is short-lived; the tree may begin to decline at 40 years of age, and it rarely lives over 100 years (Collingwood 1937). On better sites, maximum height may be 100 feet (30 m). The bark is thick 1 to 1.5 inches (2.5-4.0 cm), rough and deeply furrowed reddish brown to dark gray. Open grown trees have short boles, branching at 10 to 15 feet (3-5 m) above the ground. Young shoots are smooth, purplish-brown (Kanwar and Pamposh 2000). On good sites, however, black locust trees with longer, clear, straight trunks may be produced. The roots of black locust are usually shallow, fibrous and wide-spreading, but deep roots of up to 26 feet (8 m) or more can also be produced, especially on xeric sites. It coppices freely and produces root suckers in great abundance from its peripheral roots. Radial root spread is usually 1 to 1.5 times the tree height (Kanwar and Pamposh 2000). The leaves are 10-15 cm long, the petiole is swollen at the base, leaflets 9-19 oval or elliptic and 2.5-5.0 cm long. The leaves fold and droop with cloud cover or in the evening. The paired stipules at the base of each leaf develop into persistent woody spines. The inflorescence is a large, white, very fragrant, pendant raceme up to 20 cm long on the current year's shoots (**Fig. 2**). The fruit is a flattened legume 2 to 4 inches (5-10 cm) long. The fruit opens while still on the tree.

Fig. 2 Inflorescence of *Robinia pseudoacacia* Linn.

HABITAT

Black locust forms pure stands only on disturbed soils where there is no competing overstory vegetation. On good sites, single trees or small groups may persist and grow large enough to form part of the mature canopy. Black locust is found in the southeastern United States largely within oak (*Quercus* spp.)-hickory (*Carya* spp.) forests. It also occurs in naturalized populations in a wide range of types including blue ash (*Fraxinus quadrangulata*) savannas in the inner bluegrass region of Kentucky (Bryant *et al*. 1980). Outside of its native range, black locust often naturalizes in riparian habitats or floodplains.

GENERAL DISTRIBUTION

The original natural range of black locust is in two sections:
- the central Appalachian Mountains from central Pennsylvania and southern Ohio south to northeastern Alabama, northern Georgia, and northwestern South Carolina.

- the Ozark Plateau of southern Missouri, northern Arkansas, northeastern Oklahoma, and the Oachita Mountains of central Arkansas and southeastern Oklahoma. Outlying populations thought to be part of the original natural range occur in southern Indiana, Illinois, Kentucky, Alabama, and Georgia.

Black locust has been successfully planted in almost every state. Naturalized populations occur throughout the United States, southern Canada, Europe, and Asia (Huntley 1990).

USES

Wood

Black locust wood is strong and hard with a specific gravity of 0.68, yet it has the lowest shrinkage value of US domestic woods. The wood makes a good charcoal. Wood energy yield is typical of temperate broadleaf trees, about 19.44 x 10^6 J/kg (Stringer and Carpenter 1986). One cord of black locust wood (at 20% moisture) yields as much of heat as approximately 1.12 tons of anthracite coal (Carpenter 1981; Bongarten *et al*. 1992). The beautiful light to dark brown wood is used to make paneling, siding, flooring, furniture, boat building (substitute for teak), decking, vineyard or nursery props, fruit boxes, and pallets. It is also a preferred wood for pulp production. Black locust wood is highly resistant to rot.

Benefits to livestock and wildlife

Despite its hardwood black locust is susceptible to insects and is therefore a good host cavity tree particularly for wood peckers (Huntley 1990). Black locust has become an important tree in the Himalayas where it is heavily lopped for fodder. Leaves have a crude protein content of 24%. Black locust is rated fair in energy value and poor in protein value. However, black locust has relatively high leaf nitrogen levels. Carey and Gill (1980) rated black locust as only fair in browse value for all species of wildlife. It is planted in Europe as a nutritious livestock forage, rivaling alfalfa in nutritional value. However, tannins and lectin proteins found in leaves and inner bark can interfere with digestion in ruminants and in non-ruminants. Horses are more sensitive to the toxic substances present in various parts of plants then other livestock species. Tannin levels are high in young leaves but decrease as leaves mature. Black Locust poisoning is rarely fatal. Palatability ratings of black locust in Utah are fair for cattle and horses and poor for sheep.

Honey

Bees harvest *Robinia* nectar to produce a honey regarded as one of the world's finest. Selections of *R. pseudoacacia*, with higher sugar content of the nectar and with later and longer periods of flowering (Keresztesi 1969) have significantly increased honey-bee pastures and improved aesthetic aspects by afforestation of regions and shelterbelts.

Rehabilitation

Natural revegetation of mined sites in southwestern Virginia and northeastern Tennessee includes black locust, usually as root sprouts from adjacent forests (Muncy 1989). Volunteer black locusts can modify sites to favor forest reestablishment. Black locust vegetatively colonizes mined sites that have been reclaimed to grassland. Black locust was the most frequently and widely used tree for mine soil plantings in the United States as of 1981 (Vogel 1981). It is also planted in some areas of Ontario, Canada. Black locust is planted on mine spoils to ameliorate poor soil conditions and to stabilize mine soils and/or badly eroded or gullied land. It is adapted to a wide range of mine soil types. Its habit of sprouting has been a cause of concern where its long term occupation of a site is undesirable. Surface-mined lands that are otherwise unproductive may produce good economic returns if planted for short-rotation, woody biomass fuels. Black locust may be productive for this purpose, since it exhibits rapid early growth, and sprouts after cutting. Biomass yields were measured for black locust for various planting spacings. The tree is used extensively to rehabilitate surface mine tailings in the US.

Other

Black locust flowers have been used to make tea (Stubbendick and Conard 1989). In Hungary, black locust is often grown for wood on small private farms. A dense growth habit makes black locust suitable for windbreaks, a use most common in China. Black locust may even prove useful for alley cropping in temperate climates. Researchers at the Rodale Research Center in Pennsylvania are experimenting with intercropping black locust with vegetables. Nitrogen-fixing bacteria associated with nodules on the roots of *Robinia* increases the nitrogen content of the soil in which the tree grows. Soil calcium, magnesium, potassium, nitrates and pH increases with decomposition of the leaf litter thereby increasing the soil fertility. Mixed plantings of black locust and conifers, however, can lead to reduced growth or death of the slower growing conifers because of shading and over-topping (Kellogg 1936).

Landscape uses

For difficult sites, for shade, erosion control, flowering effect, revegetation of strip mines (Vogel 1981) as strip mine spoil banks are one of the most hostile environments for plant establishment and development, generally because of extremes in pH, texture, and slope. Soil temperature, low water-holding capacity, and nutrient status can also be limiting factors, use should be tempered in most residential landscapes; the wood is hard, making good fence posts.

REGENERATION PROCESSES

Sexual reproduction

Black locust reaches sexual maturity at approximately 6 years of age. Traditionally this tree is raised through seeds. The best seed production occurs between 15 and 40 years of age. Seed production continues until about age 60. Good seed crops are produced every 1 to 2 years. Seeds are wind dispersed and may be collected from the tree as they begin to mature. Pods are collected from the trees in September–October by hand picking or hailing or stripping or beating and thereafter spread out in the sun to dry. Seeds have a hard testa which is impermeable to water and require scarification for germination to take place. Seedlings established on good sites free of competition show rapid early growth. The best natural seed bed for *Robinia* seeds is fresh moist mineral soil under *in vivo* conditions and MS basal medium under *in vitro* conditions (Kanwar and Pamposh 2000).

Vegetative reproduction

R. pseudoacacia is a difficult-to-root species. Juvenile and mature hardwood cuttings of *R. pseudoacacia* prepared in spring season and treated with different concentrations of auxins and fungicide when planted in mist chamber showed rooting. The stem cuttings show 30-40% of rooting on treatment with 500 mg/l naphthaleneacetic acid or 750 mg/l indolebutyric acid (IBA). Grafting should be done in January and February for 45-50% success (**Fig. 3**).

Black locust produces root and stump sprouts. Sprout production is stimulated by top damage. Root suckers are usually more important to reproduction than are seedlings. Root suckers first appear when stems are 4 or 5 years old. Root suckers are produced in great abundance from its peripheral roots which extends to considerable distances even up to 12 to 15 m from the parent tree. Sprouting is an im-

Fig. 3 Rooted stem cuttings of *Robinia pseudoacacia* Linn.

portant mechanism for colonizing areas that have herbaceous plant cover but no woody canopy. Grasses form a sod that does not allow black locust seedling establishment, but black locust root sprouts are able to colonize these areas. Rooted stem cuttings can also be used for vegetative propagation of this species (Swamy *et al*. 2002).

Micropropagation

Micropropagation has shown great promise with this species using various explants such as shoot buds, nodal segments. The basis for the application of micropropagation is the capacity of plant cells and organ tissues to develop into complete plants, which can be grown to maturity. Micropropagation generally involves four steps (**Fig. 4**):

1. initiation of aseptic cultures;
2. shoot multiplication;
3. rooting of *in vitro* formed shoots;
4. transplantation of plants to a potting mixture and thereafter in field conditions.

Kanwar *et al*. (1996) used terminal and axillary buds as explants and established them on MS medium (Murashige and Skoog 1962) supplemented with benzylaminopurine (0.2-1.0 mg/l) and naphthalene acetic acid (0.05 mg/l). The best establishment of the explants was observed in March-April which corresponds to the end of dormancy period (Kanwar *et al*. 1997). It was reported that the age of plants from which explants were taken had a marked influence on its establishment. The explants from 2-year old plants showed maximum percentage of shoot establishment as compared to a 12-year old tree (Kanwar *et al*. 1995). The *in vitro*-raised seedlings were isolated and dipped in IBA for 24 hours under dark and aseptic conditions, thereafter cultured on hormone-free half strength solid MS medium for 4 weeks (**Fig. 5**). Plantlets were transferred into pots containing soil taken from a *Robinia* plantation to induce nodules before transferring to field conditions.

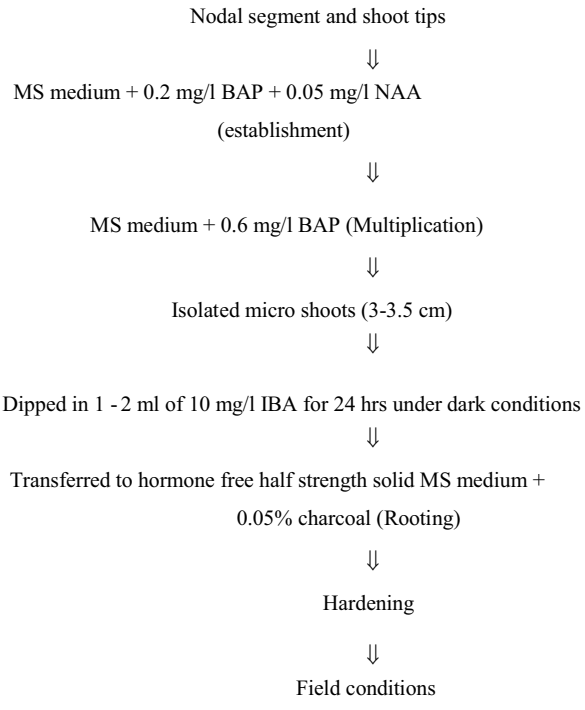

Fig. 4 Protocol for micropropagation of *Robinia pseudoacacia* Linn.

Fig. 5 *In vitro* raised plantlets of *Robinia pseudoacacia* Linn.

Assessment of micropropagated plantlets

As a consequence of *in vitro* culture, various genetic, cytological and phenotypic variabilities, collectively known as somaclonal variation (Larkin and Scowcroft 1981) have frequently been observed in regenerated plants. Allozyme markers can be used for examining cryptic somaclonal variations. DNA markers are a more attractive means for examining genetic similarity/dissimilarity. Technologies such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNAs (RAPD) and now Amplified Fragment Length Polymorphism (AFLP) allow geneticists and plant taxonomists to look directly at the genotype of a plant. Molecular or DNA-based markers offer many more advantages over conventional phenotypic markers. They are; 1) detectable in all tissues; 2) developmentally stable; 3) unaffected by environmental situations and 4) provide a choice of dominant or codominant markers. RAPDs have been applied in the characterization of micropropagated forest trees (reviewed in Teixeira da Silva *et al*. 2005. Kaushal and Kanwar (2003) employed RAPD markers to determine the genetic homogeneity/somaclonal variations in a small sample of micropropagated plants (4-years old) of *R. pseudoacacia*. The results showed that somaclonal DNA sequence variations are present even when organized cultures such as shoot buds are used as explants for micropropagation.

Protoplast isolation and culture

Protoplast culture has now become a baseline for the successful genetic improvement of many forest species such as *Robinia*, *Populus*, and *Eucalyptus*. Various plant tissues are used but leaf tissues are the most frequently used source in many crops, including forest trees. Cotyledons have also been used as a source tissue for protoplast isolation. At the same time specific plant growth conditions are necessary for successful plant regeneration from the protoplasts so isolated. The removal of the cell wall, while conserving the cytoplasmic and nuclear constituents of the cell necessary for the cell wall regeneration and cell division, also leaves the plasma membrane exposed as the only barrier between the interior of the totipotent cell and the external environment. Such as an easy accessibility facilitates genetic manipulation and gives ample scope to use this technique in crop improvement programmes. Studies were conducted to isolate and purify protoplasts from leaf mesophyll cells of *in vitro* raised microshoots of *R. pseudoacacia* in order to regenerate whole plants (Bharadwaj 1999). Cellulase and maceroenzyme in combination with concentrations varying from 1.0-2.0% and 0.1-0.5%, respectively were used for protoplast isolation. The leaf segments were incubated for 12-48 hrs in different enzyme mixtures. The crude suspendsion thus obtained was filtered and centrifuged at 500 rpm for 10-15 minutes. The supernatant was mixed with cell protoplast washing (CPW) solution and centrifuged at 10,000 rpm for 3-4 minutes. Protoplasts settled as a pellet. The pellet was resuspended in CPW and again centrifuged. The protoplast pellet was resuspended in liquid Nagata and Takebe's (NT) basal medium (1971). The isolated protoplasts were plated on solid NT medium supplemented with 5 µM NAA and 1 µM 6-benzylamino purine (BAP). Microcalli visible to the naked eye were transferred to MS medium supplemented with 5 µM NAA and 5 µM BAP. The pieces of callus were trans-ferred to shoot regeneration medium (MS medium + 0.5 µM NAA + 1.0 µM BAP). Well developed shoots were transferred to rooting medium (1/2 MS medium + 0.05% activated charcoal (AC)).

Cell suspension culture

Plant cell culture facilitates the rapid production of variant cell lines via selection procedures, very similar to those employed in microbial system. Callus tissue is an essential material in plant cell culture systems. When it is agitated into a liquid medium, the cells disperse throughout the liquid to form a suspension culture. Such cells are in theory totipotent and should also have the potential to synthesize any of the compounds normally associated with an intact plant. Cells in suspension exhibit much higher rates of cell division than those in callus culture. Thus, cells in suspensions offer advantage when rapid cell division or many cell generations are desired. Cell suspensions have also proven to be excellent starting materials for the isolation of protoplasts to be used in a wide range of applications including cell fusion and genetic manipulation.

Kanwar *et al*. (2007) developed a protocol for plant regeneration of *R. pseudoacacia* from cell cultures:

- *In vitro* seed germination on MS basal medium;
- Induction of callus from cotyledon explants on MS medium supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D);
- Initiation of cell suspension culture from friable callus on MS liquid medium supplemented with 0.5 mg/l 2,4-D;
- Isolation of single cells after 3 days of incubation at 21 ± 2°C and 100-120 rpm;
- Plating of single cells on MS medium supplemented with 0.5 mg/l 2,4-D and 0.6% agar;
- Shoot regeneration and multiplication on MS medium supplemented with 0.05 mg/l NAA and 0.6 mg/l BAP;
- *In vitro* rooting on MS medium supplemented with 0.05% AC.

DISEASES AND PESTS

Diseases

Heart-rot

It is caused by *Trametes robiniophila* and *Fomes rimosus*. Large yellowish sporophores are observed on tree infected with white heart rot (Kauffman and Kerber 1992).

Witches' broom

Light and transmission electron microscopy of phloem sieve-tube elements, companion cells, and parenchymal cells in thin and ultra thin sections of small and medium rachises and small, medium and large leaflets of a black locust tree affected by witches' broom disease revealed (in the small and medium rachises and leaflets) structures that were characteristic of phytoplasmas, and crystal-like inclusions in the phloem sieve-tube members. A crystal-like inclusion was also seen in a companion cell. Results from analysis of 16S rRNA gene sequences amplified by the polymerase chain reaction indicated for the first time that the phytoplasma associated with black locust witches' broom is a member of group 16 SrIII (peach X-disease) phytoplasma group (Campman *et al*. 2001).

Verticillium or Fusarium Wilt

The fungus damages water conducting mechanism and plant wilts. Over-fertilization can worsen this problem. Practicing crop rotation and removing infected plants by pruning can be effective control of this problem.

Insects

The most serious pest to black locust in the US is the locust borer, *Megacyllene robiniae* (Forster). The locust borer causes severe damage to form, wood quality and overall vigor in many plantations. Older trees that are already low in vigor are particularly susceptible to locust borer infestation (Huntley 1990). There is some evidence for genetic resistance to the borer. Cut worms, white grubs and termites cut the seedling at the nursery stage during night time. Another insect confined to trees in the US is the locust twig borer, *Ecdytolopha insiticiana* (Zeller). Leaf miners attack the young leaves in spring season. *Myllocerus* beetle attacks the scented flowers which results in low seed set (Anderson 1981).

TREE IMPROVEMENT

R. pseudoacacia has been cultivated for over 350 years. Natural variation in numerous traits has often been observed and many cultivators described. Most of the diversity resided within seed sources with low geographic variation. Cultivators vary in crown and stem form, growth rate, growth habit (upright vs. prostrate), leaf shape, thorniness, flowering characteristics, and phenology. Comprehensive germplasm collections and plantings for provenance tests were begun in 1982 at Mich. State Univ. Efforts in crossbreeding were under way to improve the tree for growth rate, borer resistance, stem form, thorn-lessness, or other traits (Hanover *et al*. 1989). In Hungary, a large array of tall clones is in commercial use, based on seeds from trees of "shipmast locust" originating from Long Island in New York State. The improvement by conventional tree improvement programmes is cumbersome and time consuming. *In vitro* methods offer an alternative approach to overcome these constraints.

Genetic transformation

Genetic transformation is a particularly attractive approach of plant improvement especially for those species which are

vegetatively propagated. The major advantage is the potential to add a character directly to a proven genotype. *Agrobacterium*-mediated genetic transformation of *R. pseudoacacia* could contribute towards expansion of the genepool of the species by allowing introduction of useful genes. Davis and Keathley (1989) were the first to report that *R. pseudoacacia* was a host for *Agrobacterium tumefaciens* and *A. rhizogenes*. They also concluded that *Agrobacterium* could be used to introduce a selectable marker (Kanamycin resistance gene) into black locust cells. Later Han *et al*. (1993) worked on regeneration of transgenic *R. pseudoacacia* and morphological alteration induced by *A. rhizogenes*-mediated transformation. Kanwar *et al*. (2003) conducted a set of experiments to establish a successful transformation procedure along with regeneration of transformed tissue of *R. pseudoacacia*. *Agrobacterium* strain LBA4404 harbouring a binary vector (pBI121) that contained the chimeric neomycin phosphotransferase II (NPT II) and β-glucuronidase (GUS) gene was co-cultivated with hypocotyl segments of *in vitro*-raised seedlings of *Robinia*. A transformation frequency of 16.67% was obtained by 48 hr of pre-conditioning followed by 48 hr of co-cultivation. Transformed tissue was selected by the ability to grow on 50 mg/l kanamycin containing medium. Successful regeneration was followed after histochemical GUS assay for the detection of transgenic tissue. No other molecular confirmation methods were employed. The protocol for *Agrobacterium*-mediated genetic transformation of *R. pseudoacacia* using hypocotyl segments involved the following steps:

- *In vitro* seed germination (same as above);
- Pre-conditioning of hypocotyls segments on callus induction medium (MS medium + 5 µM NAA + 10 µM BAP);
- Co-cultivation of 10-15 days old hypocotyl segments with *Agrobacterium* strain containing the desired gene (GUS) to be transferred;
- Selection of transformed cells on selective callus induction medium (MS medium + 5 µM NAA+ 10 µM BAP + 50 mg/l Kanamycin+ 500 mg/l Carbenicillin);
- Callus initiation and multiplication on selective callus induction medium;
- Histochemical β-glucuronidase assay.

IN VITRO NODULATION

Robinia is fairly specific in its *Rhizobium* requirements. Although it will form nodules with a variety of exotic strains, for effective N-fixation, strains from native trees work best. Newly introduced trees require inoculation; inoculum may be retrieved from the soil of black locust stands, or from Nitrogen Fixing Tree Association or NFTA, USA. The tree's fine roots are also colonized by VA mycorrhizae. *In vitro* nodulation of micropropgated plants of a legume by *Rhizobium* was first reportd in *Leucaena leucocephala* by Dhawan and Bhojwani (1987). Efforts have been made by Kanwar and coworkers in 1998 for *in vitro* nodulation of micropropagated *R. pseudoacacia* during the *in vitro* rooting and hardening phase. The various steps involved in *in vitro* nodulation (**Fig. 6**) of micropropagated plantlets of *R. pseudoacacia* are:

- collection of healthy, pink-coloured nodules from seedlings growing among natural plantations;
- crushing the nodule in a drop of sterile water (500 mg nodule + 2 ml sterile water);
- take a loopful of the above in an inoculating loop and perform a streak on solid Yeast Extract Mannitol (YEM) agar medium;
- purified *Rhizobium* isolate obtained by repeated streaking (2-3 times; **Fig 7**);
- inoculate a loopful of above in 100-150 ml YEM broth, incubated at 28°C on a rotary shaker at 64 rpm for 3-4 days;
- centrifuge and resuspend pallet in nitrogen-free nutrient solution (it contains macro and micronutrients of MS basal medium excluding vitamins, nitrates of ammonium and potassium and sodium EDTA);
- inoculate microshoots (kept in double-sterilized sand) at 2-3 ml of freshly prepared bacterial suspension has to be added to each flask 4-5 times at weekly intervals;
- after 40-45 days loosen plugs;
- transfer plantlets to pots.

Fig. 6 Comparison of root system after (left) *Rhizobium* inoculation.

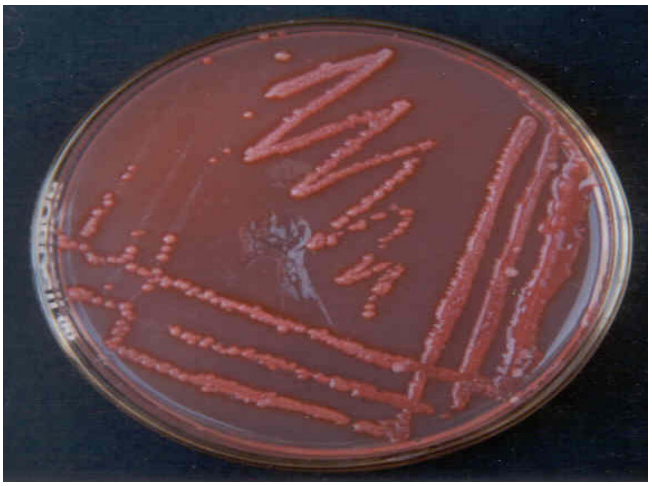

Fig. 7 *Rhizobium* colonies on Yeast Extract Mannitol Agar (YEMA) medium.

CONCLUSIONS AND FUTURE PERSPECTIVES

Robinia pseudoacacia L. is a nitrogen-fixing and multipurpose tree. There is a need for expanding and taking advantage of biotechnological tools for the improvement of this tree. Micropropagation techniques for this species have already been standardized and *in vitro* plants can be produced from the elite trees; this seems to be promising in the demonstration plot under field conditions, however hardening still needs refinement in the technology for higher survival of plantlets. *In vitro Rhizobium* inoculation improves the hardening during acclimatization and the chances of survival on field transfer from tube to *ex vitro* conditions. Improvement in the *Rhizobium* strains may help in further improving the technology.

According to studies on host range, *Agrobacterium* can infect a wide spectrum of woody plants, including *R. pseudoacacia*. However, until recently there were few reports demonstrating *Agrobacterium*-mediated genetic transformation of *R. pseudoacacia*, which could contribute a great deal towards the improvement of this species.

REFERENCES

Anderson RL, McClure JP, Hoffard WH, Cost ND (1981) Incidence and impact of damage to South Carolina's timber, 1979. Resource Bulletin, U.S. Department of Agriculture, Forest Service, Southeastern Forest Experiment Station, Asheville, NC, 34 pp

Bhardwaj A (1999) Studies on *in vitro* cultured protoplast of *Robinia pseudoacacia* L. PhD Thesis, Dr. YS Parmar University of Horticulture and Forestry, Solan, 131 pp

Bongarten Bruce C, Huber Dudley A, Apsley David K (1992) Environmental & genetic influences on short-rotation biomass production of black locust (*Robinia pseudoacacia* L.) in the Georgia Piedmont. *Forest Ecology and Management* **55**, 315-331

Bryant WS, Wharton ME, Martin WH, Varner JB (1980) The blue ash-oak savanna-woodland, a remnant of presettlement vegetation in the Inner Bluegrass of Kentucky. *Castanea* **45**, 149-165

Chapman GB, Buerkle EJ, Barrows EM, Davis RE, Dally EL (2001) A light and transmission electron microscope study of a black locust tree, *Robinia pseudoacacia* (Fabaceae), affected by witches'-broom, and classification of the associated phytoplasma. *Journal of Phytopathology* **10**, 589-597

Carey Andrew B, Gill John D (1980) Firewood and wildlife. Res. Note 299. U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station, Broomall, PA, 5 pp

Carpenter SB (1981) Biomass planting for energy production on reclaimed surface mines. In: *Land-use Allocation: Processes, People, Politics*, The Society of American Foresters, Washington, DC, pp 240-242

Collingwood GH (1937) *Knowing Your Trees*, The American Forestry Association, Washington, DC, 213 pp

Davis JM, Kethley DE (1989) Detection and analysis of T-DNA in crown gall tumors and kanamycin resistant callus of *Robinia pseudoacacia*. *Canadian Journal of Forestry Research* **19**, 1118

Dhawan V, Bhojwani SS (1987) *In vitro* nodulation of micropropagated plants of *Leucaena leucocephala* by *Rhizobium*. *Plant and Soil* **103**, 274-276

Han KH, Keathley DE, Davis JM, Gordon MP (1993) Regeneration of a transgenic woody legume (*Robinia pseudoacacia* L., black locust) and morphological alterations induced by *Agrobacterium rhizogenes*-mediated transformation. *Plant Science* **88**, 149

Hanover JW, Mebrahtu T, Bloese P (1989) Genetic improvement of black locust: A prime agroforestry species. Proc. First Conf. on Agroforestry in N. America, Aug. 1989, Guelph, Ontario, Canada

Harlow WM, Harrar ES, White FM (1979) *Text Book of Dendrology* (6th Edn), McGraw Hill, New York, pp 391-399

Huntley JC (1990) *Robinia pseudoacacia* L. black locust. In: Burns R M, Honkala B H (Tech. coordinators) *Silvics of North America (Vol 2) Hardwoods, Agricultural Handbook 654*, U.S. Department of Agriculture, Forest Service, Washington, DC, pp 755-761

Kanwar K, Bhardwaj A, Nath AK, Pamposh (1998) *Rhizobium* inoculation in micropropagated *Robinia pseudoacacia* L. for improving hardening. Tree Science Conference, April 10-13, 1998, New Delhi, p 48

Kanwar K, Bhardwaj A, Aggarwal S, Sharma DR (2003) Genetic transformation of *Robinia pseudoacacia* by *Agrobacterium tumefaciens*. *Indian Journal of Experimental Biology* **41**, 149-153

Kanwar K, Pamposh, Khosla PK (1996) Mass propagation of *in vitro* raised plants of thornless strains of *Robinia pseudoacacia.* QFRI-IUFRO Conference 27th Oct.-1st Nov. 1996, Queensland, pp 518-519

Kanwar K, Pamposh, Prasad S (1997) Seasonal effect on *in vitro* propagation of *Robinia pseudoacacia*. *Annals of Forestry* **5**, 107-110

Kanwar K, Pamposh (2000) Propagation strategies for *Robinia pseudoacacia* L. (black locust). In: Kohli RK, Singh HP, Vij SP, Dhir KK, Bakshi DR, Khurana DK (Eds) *Man and Forests*, DNAES, IUFRO, ISTS, pp 197-212

Kanwar K, Sehgal RN, Sood D (1995) Effects of explants type on the micropropagation of *Robinia pseudoacacia*. *Indian Journal of Forestry* **18**, 47-52

Kaushal B, Kanwar K (2003) Random amplified polymorphic DNA (RAPDs) markers for genetic analysis in micropropagated plants of *Robinia pseudoacacia* L. *Euphytica* **132**, 41-47

Kanwar K, Kaushal B, Abrol S, Raj Deepika (2007) *In vitro* regeneration from cell suspension culture in *Robinia pseudoacacia* L. *Biologia Plantarum*, in press

Kanwar K, Kaushal B, Sharma DR (2002) Isozyme variation in micro and seed propagated plants of *Robinia pseudoacacia* L. *Journal of Tree Science* **1/2**, 46-51

Kartesz JT, Kartesz R (1980) *A Synonymized Checklist of the Vascular Flora of the United States, Canada, and Greenland (Vol II) The Biota of North America*, The University of North Carolina Press, Chapel Hill, NC in confederation with Anne H. Lindsey and C. Richie Bell, North Carolina Botanical Garden, 500 pp

Kellogg LF (1936) Failure of black locust-coniferous mixtures in the Central States. Station Note No. 15. Columbus, U.S. Department of Agriculture, Forest Service, Central States Forest Experiment Station, Ohio, 4 pp

Kereszteski B (1969) Selection of forest trees and shrubs for improvement of the bee pastures and in favour of the landscape architecture. Second World Consult. Forest Tree Breeding. FAO-FO-FTB-69-6/5

Kereszteski B (1981) The black locust. *Unasylva* **32**, 23-33

Kauffman CH, Kerber HM (1922) A study of the white heart-rot of locust, caused by *Trametes robiniophila*. *American Journal of Botany* **9**, 493-508

Larkin P, Scowcroft N (1981) Somaclonal variation – a novel source of variability from cell for plant improvement. *Theoretical and Applied Genetics* **60**, 197-214

Little Elbert L Jr. (1979) Checklist of United States trees (native and naturalized). Agricultural Handbook 541, U.S. Department of Agriculture, Forest Service, Washington, DC, 375 pp

Muncy JA (1989) Reclamation of abandoned manganese mines in southwest Virginia and northeast Tennessee. In: Walker DG, Powter CB, Pole MW (Eds) *Reclamation, a Global Perspective*, Proceedings of the conference, 1989 August 27-31, Alberta Land Conservation and Reclamation Council, Calgary AB, Edmonton AB, pp 199-208

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Muthoo MK, Kango GH (1965) *Robinia pseudoacacia* in India with special reference to Jammu and Kashmir. *Indian Forester* **91**, 126-130

Nagata T, Takebe I (1971) Plating of isolated tobacco protoplast mesophyll protoplasts on agar medium. *Planta* **99**, 12-20

Stringer JW, Carpenter SB (1986) Energy yield of black locust biomass fuel. *Forest Science* **32**, 1049-1057

Stubbendiek J, Conard EC (1989) *Common Legumes of the Great Plains: An Illustrated Guide*, University of Nebraska Press, Lincoln, NE, 330 pp

Swamy SL, Puri S, Kanwar K (2002) Propagation of *Robinia pseudoacacia* Linn. and *Grewia optiva* Drummond from rooted stem cuttings. *Agroforestry Systems* **55**, 231-237

Teixeira da Silva JA, Nhut DT, Giang DTT, Rashid SZ (2005) The phylogeny, breeding and ecology of trees using molecular markers. In: Thangadurai D, Pullaiah T, Tripathy L (Eds) *Genetic Resources and Biotechnology* (Vol III), Regency Publications, New Delhi, India, pp 278-308

Troup RS (1921) *The Silviculture of Indian Trees* (Vol 1), Clarendron Press, Oxford, pp 332-336

Vogel WG (1981) A guide for revegetating coal minespoils in the eastern United States. Gen. Tech. Rep. NE-68. U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station, Broomall, PA, 190 pp