Micropropagation of *Robinia pseudoacacia* L. is base for efficient breeding work

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Summary: Black locust (*Robinia pseudoacacia* L.) is one of the most important broadleaved trees in Hungary in spite of it is not a native tree in the Carpathian basin. Selection of them is going on from the early fifties in the Hungarian Forest Research Institute to improve the quality of black locust forests as well as plantation for industrial use. The quick changes of the selection's aim has to be followed by the method of propagation. Lack of efficient conventional vegetative propagation method for *Robinias* resulted in the collaboration between the Hungarian Forest Research Institute and Micropropagation Laboratory of the Research Institute for Fruitgrowing and Ornamentals many years ago to develop the procedure of micropropagation.

Actively growing juvenile shoot tips are most convenient to initiate sterile cultures. Following repeated disinfections the shoot-tips are directly put on modified MS propagation medium into the culture room. The shoot proliferation starts about 4-5 weeks following the initiation of the culture and three month later the usual propagation rate of the variety is obtained. The newly grown shoots can be rooted in a half strength modified MS medium containing growth regulators depending on the clone. The rooted plantlets can be acclimated under greenhouse conditions and 6 weeks later transplanted outside if provided with an adequate shading and watering system.

Key words: black locust, broadleaved trees, *in vitro*, selected clones, vegetative propagation

Introduction

Black locust is one of the most widespread species among the fast growing forest trees in Hungary. As the aim of the breeding or selection changes rather quickly, market demands has to be followed by the method of propagation. *In vitro* multiplication of the newly selected clones is a suitable process for speeding up the production.

Robinia pseudoacacia was used as a test-plant for physiological studies by *Jacquiot* (1951, 1964) and for the solution of ontogenetic problems by *Trippi* (1963). *Seelinger* (1956, 1959), who worked with isolated roots of *Robinia pseudoacacia* observed shoot initiations on the cultured roots. *Brown* (1980) in the USA was the first to report successful *in vitro* method for mass production of black locust. *Chalupa* (1983) in Bohemia, *Enescu* and *Jucan* (1985) in Romania started experiments in view of similar results. *Balla* and *Vértesy* in 1985 had the first success in the sterile production of four Hungarian varieties of Keresztesi. *Barghchi* (1987) has been developed the procedures for micropropagation of the Hungarian originated cv. 'Jászkíséri' in New Zeeland. *Davis* and *Keathley* (1987) found big difference during the establishment of *in vitro* culture originated from mature tree. *Han* et al. (1993) detected tree specific shoot regeneration capacity on cambial originated callus. Buds grown on top branches of mature trees were not recalcitrant in terms (*Han* et al. 1997) of morphogenetic capacity.

Balla et. al (1998) published the improvement of the acclimation results of micropropagated black locust using symbiotic microorganisms. Their results was strengthen by *Tian* et al. (2003), whose group used ecto- and arbuscular mycorrhizae together with *Rhizobium* for inoculation of black locust seedlings under *in vitro* conditions.

As far as the first micropropagated black locust plantlets were produced question of genetic uniformity of the only clones as well the possibility to distinguish the *in vitro* propagated

clones/cultivars by molecular methods arisen. *Major* et al. (1998) could surely separate eleven genotypes from the examined twelve ones by RAPD markers. Interest in micropropagation and biotechnology as well as genetic control of black locust plants produced *in vitro* of late years is very keenly in Far East, in China and Korea, which is supported with several publications in the topic (*Bindiya - Kanwar* 2003., *Shu* et al. 2003., *Ngezahayo* et al. 2006., *Guo* et al. 2006.). Low level of genetic modification could be detected by ISSR markers in case of bud originated multiplication in contradiction to callus originated shoot regeneration. All of this changes has no observable morphologic variation.

Materials and methods

Plant material

More than hundred new varieties or selected clones were propagated during the last few years in the Micropropagation Laboratory of the Research Institute for Fruitgrowing and Ornamentals, Budapest in a collaboration with the Hungarian Forest Research Institute, Budapest.

All the explants originated from the experimental field of the Hungarian Forest Research Institute.

Micropropagation

Propagation under *in vitro* conditions is the most suitable method to follow the results of the breeding work. Collecting some shoots with buds for explants of the production does not cause any damage on the selected tree and can be a base for mass production. It can assure in shorter time to produce planting material for mother plantation as well as for experimental field or for reforestation. It is important, because in the near future thousands of hectares will be afforested.

Steps of micropropagation procedure were developed for *in vitro* production of black locust clones/cultivars.

Results and Discussion

Culture establishment

Shoot cultures could be initiated most conveniently from actively growing juvenile shoots - e.g. from vegetative stool-beds, but most of actively growing shoot tips of the varieties propagated were collected from adult trees, standing in a collection of varieties of the Forest Research Institute. May and June, and perhaps September is the most suitable time for culture initiation. The shoot-growth is most active in this period of the year. In springtime the disinfections does not mean difficult problems, growing open ground, without any plant-protection.

Shoot tips of 10 - 15 mm in length, having apical- and lateral buds, without any leaves survive the repeated disinfections in 1 % $HgCl_2$ solution containing a few drops of wetting agent /Tween 80/ and are not damaged if properly rinsed with sterilized, bidestillated water. The disinfected shoot tips are directly put on the proliferation medium, where about 70 % of the cultures are found uncontaminated.

Multiplication under in vitro conditions

Black locust naturally looks to be very sensitive to chemicals, contaminations. The same opinion was found during the *in vitro* propagation. Shoot multiplication was optimal on Murashige - Skoog medium (1962), but to minimize profuse callus production some modifications were necessary. The concentration of major and minor elements had to be lowered - except of $MgSO_4$ - and NH_4^+ and Cl^- have to be omitted. Oxidation of the shoot basis was diminished by the addition of ascorbic acid. Proliferation was enhanced by a

relatively high sugar concentration. Cytokinins and auxins were applied at a concentration of 10^{-9}. Optimal multiplication rate was detected beside proper elongation of the new shoots under combination of BAP and adenine-sulphate. The medium is solidified with 0.6 % of agar-agar and the pH is adjusted to 5.2.

Cultures are kept in commercially used jars of 220 ml. The culture vessels are closed with semipermeable, selfclinging polyethylene film. The cultures are placed in an air-conditioned room at 22 °C, under a light intensity of 2000 Lux /Tungsram tubes, type F-29/ with a photoperiod of 16/8 h.

Shoot proliferation actually started 4 - 5 weeks after initiation of the culture, when the shoots are subcultured to the same fresh medium. The lateral buds begin to grow and develop into shootlets. 2 - 5 new shoots appear monthly at the basis of the older ones. The multiplication ratios are different depending on the growth vigour of the cultivars.

Rooting in vitro

10 - 15 mm long shoots are selected for rooting. Rooting of black locust clones is successful on half – strengths MS medium supplemented with auxines according to the demand of the clone. The rate of root formation is 60 to 90 % depending on the cultivar.

Acclimatization

Acclimation of the black locust clones are performed under greenhouse conditions. The rooted plants are transplanted into a mine sterile substrate. The *ex-vitro* plants need a relatively dry substrate and a high relative humidity to avoid microbial diseases. The glasshouse cabinet used for acclimation purposes should be shaded during the summer months. Temperatures above 30 °C and below 15 °C may be equally disastrous.

Black locust plantlets root thoroughly into the substrate and start to grow vigorously. The survival rate during the acclimatization is about 70-80 % and can be enhanced with artificial microbial inoculation. Plants may be cultivated further in plastic containers or even in soil under field -conditions if provided with an adequate shading and watering system. Their soil requirement is similar to that of seedlings or root cuttings.

Application, conclusions

Micropropagation can be done theoretically all year round, nevertheless it is not advisable to initiate shoot cultures from dormant trees, to acclimatize plants in short-day conditions and keep acclimatized plantlets in the glasshouse for wintering. Glasshouse over-wintering is not only a highly cost-raising procedure, but results also in poor growth in the subsequent season, as micropropagated plantlets have in general an acute cold requirement.

It is possible to produce about 10 000 plantlets in one year out of 100 cultured shoots, initiated in May, if a 3-fold multiplication rate is taken into account, 80 % of them are actually rooted, further on where 80 % of the plantlets survive the acclimation.

Plantlets, which are acclimatized till middle of May in the greenhouse and a month later transplanted into plastic containers of 3 l outside, under shading and watering conditions, produce 1,5 m high trees that can be planted into their final orchard in November.

In case of a later acclimation period, the best solution for the over-wintering outside is covering the containers with straw.

Micropropagated black locust trees till now are used for stool-beds in Hungary and the trees for reforestation are propagated from stool-beds with traditional methods, like stem- or root-cuttings.

In spite of the many publications only one is speaking about really *in vitro* produced and grown, controlled under field condition 13,000 black locust trees (*Guo* et al. 2006).

The developed efficient micropropagation procedure of black locust in Hungary is used first of all for experimental plant production. Beside the mother plantations in the year 2000 an experiment was planted to compare 9 selected, micropropagated clones (*Rédei* et al. 2001). Their morphological habit and uniform growth assure the reason of the existence of micropropagation in forest-tree production.

The productivity of somatic embryogenesis is higher than the *in vitro* multiplication, and this method is used for *in vitro* production of *Conifers*. Trials for regeneration of black locust via somatic embryogenesis were succeeded by *Merkle* and *Wiecko* (1989), but the procedure was not spread.

In the future *in vitro* culture can become a base for breeding work using molecular methods, like genetic transformation, as it was published by *Han* et al. (1993).

Health of the forest-trees, similarly to the fruit-trees have more and more importance so as like using genetic transformation to improve resistance or control to detect or eliminate viral infections under *in vitro* conditions.

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