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IN VITRO CULTURE AND PROPAGATION OF *PUERARIA HIRSUTA* (THUNB.)

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Explants collected from a specimen grown in Belgrade Botanical garden, comprising lateral buds, single node segments, and pulvinii were cultured on a modified MS (Murashige & Skoog, 1962) medium supplemented with 0-1.0 mgl^{-1} BA and NAA. Explants produced only fast growing callus in which shoot differentiation could not be induced. Highest callus proliferation was registered on media with 1.0 mgl^{-1} BA and 1.0 mgl^{-1} NAA. From a plant growing near Dubrovnik, 60 large seeds were collected and aseptically germinated on MS medium with 0.5 mgl^{-1} BA and 0.1 mgl^{-1} IBA. Only two seeds germinated but both shoot explants perished after subculturing. Another seed reacted with a two month delay, producing callus which differentiated shoots. From this shoots a clone of shoot cultures was established and maintained on medium with 0.1 mgl^{-1} BA and NAA. Shoots rooted on hormone free medium could be successfully planted *ex vitro* and further cultured in glasshouse.

Key words: *in vitro,* propagation, callus, shoot cultures, compound leaves, *Pueraria hirsuta* (Thunb.).

Ključne reči: *in vitro,* razmnožavanje, kalus, kulture izdanaka, složeni listovi, *Pueraria hirsuta* (Thunb.).

INTRODUCTION

Pueraria hirsuta Thunb., member of the legume group is a sub-tropical semi-hard liana, which survives winter in the continental climate of Belgrade region. Due to the absence of specific pollinators, plant fails to develop viable seeds in Belgrade. *P. hirsuta* is characterized by very large compound leaves consisting of three individual leaflets, each equipped with an individual pedicel and a prominent pulvinus. These compound leaves can perform complex movements including sun tracking (whole leaf) and spatial rearrangement of lamina position (individual leaflets) induced by changes in irradiance. *P. hirsuta* has no suckers or tendrils but it can climb using other trees for support. It is a fast growing plant in which the main runner elongates more then 40 cm/per day under favorable conditions (D. Vinterhalter, unpublished). In the climate of Belgrade region *P. hirsuta* can be used as fast growing ornamental plant which can provide shade for gardens, veranda (porches), loggia, balconies, etc. Vegetative propagation can be performed by runner layering but this is a slow and often unreliable method. Therefore, *in vitro* propagation of this species has been investigated with intention to find a simple and fast procedure for propagation. Special attention was paid to the development of compound leaves and beginning of sun tracking movements.

The legume group contains a number of species which are difficult to regenerate and propagate by *in vitro* methods. Among them are species from genera *Phaseolus* (Allavena, 1983), *Glycine* (Newell & Luu, 1986; Hammat et al., 1986; Grant, 1984) and *Lathyrus* (Gharyal & Maheshwari, 1983). In some species there is a strong influence of genotype on the success of regeneration/propagation as for instance in *Phaseolus vulgaris* (Martins & Sondhall, 1984) and *Trifolium pratense* (Campbell & Tomes, 1984). In leguminous trees regeneration and propagation is difficult (Lakhsmi Sita et al., 1986) and these species have been characterized as recalcitrant (Tomar and Gupta, 1988).

MATERIAL AND METHODS

Material used for investigation was collected from two trees, one growing in the Belgrade Botanical garden and the second in Mlini (Dubrovnik) obtained as a cutting from the plant in arboretum Trsteno (Dubrovnik). Flowers of the plant from Mlini are visited by bumblebees and large seeds develop in some pods. These seeds were not observed to germinate under normal conditions. Sampling was performed in 1986-1987 and the resulting shoot cultures were maintained until autumn 1991.

Seeds and various plant explants were surface sterilized for 20 min in 10% commercial bleach containing 4-5% NaOCl and then thoroughly rinsed in autoclaved water. Medium contained Murashige and Skoog (1962) inorganic salts, vitamins and inositol was modified by increasing vitamin B1 concentration to 0.4 mgl^{-1} and providing 2% sucrose and 0.64% agar. Medium pH was adjusted to 5.8 prior to autoclaving which was performed for 20-25 minutes at 114-115°C.

Callus induction was performed in Ø 20 x 100 mm test tubes, and aseptical seed germination and rooting in Ø 18 x 180 mm test tubes. Shoot cultures were maintained in 100 ml wide neck Erlenmeyer flasks. All culture vessels were closed with cotton wool plugs.

Conditions in the growth room were: photoperiod 16/8 hours light to darkness, provided by cool white fluorescent lamps, irradiance 5.0-7.2 Wm^{-2} and temperature 25 ± 2°C.

RESULTS AND DISCUSSION

First round of investigation (1986) was started with explants collected from the plant growing in Belgrade Botanical garden. A total of 73 explants (21 lateral shoot buds, 36 node segments and 16 leaf pulvini) were placed on medium supplemented with 1.0 mgl^{-1} BA and 0.1 or 1.0 mgl^{-1} NAA.

All primary explants produced brownish-green callus which was very soft and friable. Callus proliferation was much better on media with 1.0 than on 0.1 mgl^{-1} NAA. It develop from cut surfaces quickly engulfing the whole explant. Most callus was produced on nodal explants and least on pulvini. Only four out of 73 explants were rejected due to contaminations.

Reaction of explants comprising lateral shoot buds was unusual. Axillary buds failed to elongate. In some explants development of vitrified leaves was promoted. Development of axillary but and leafs could not be followed due to rapid proliferation of callus which engulfed the whole explant.

In explants consisting of node explants callus proliferation was so fast that the first subculture was performed only a week after the explants were placed on midium. Subsequent subcultures were also short, not exceeding three week intervals.

Attempts to induce differentiation and organogenesis in this callus were not successful. Various concentrations of BA and NAA same as KIN and IAA or IBA were tried non of which rendered a reliable protocol for differentiation of either shoots or roots. Only two shoots differentiated one on media supplemented with 1.0 mgl^{-1} BA and 0.1 mgl^{-1} NAA and the second on media with 1.0 mgl^{-1} BA and 0.05 mgl^{-1} IBA. Both shoots could not be further multiplied on same type of media. Furthermore, leaves growing on these shoots in contact with medium became vitrified and proliferated masses of undifferentiated callus.

After a year further cultivation of this callus clone was stopped. Two main observations were made:

– fast proliferating, undifferentiated callus was friable. It appeared both on media with high and low hormone concentrations. Thus medium containing 0.2 mgl^{-1} both BA and NAA enabled rapid callus prolifetration same as medium with 1.0 mgl^{-1} BA and 2.0 mgl^{-1} NAA.

– on media supplemented with 0.1 mgl^{-1} BA and auxins at low concentration (- $\approx$ 0.1 mgl^{-1} IBA or NAA) callus manifested signs of organ differentiation including; slow growth rate, appearance of green colour, change of consistency from soft and friable to hard and compact, and finally formation of various green surface structures. In case of *P. hirsuta* these structures did not develop further into shoots.

Second round of investigation was performed with seeds collected from the plant growing in Mlini. From a sample containing 200 fresh seeds 60 largest were surface sterilized and placed on media with 0.5 mgl^{-1} BA and 0.1 mgl^{-1} IBA. After three weeks two seeds germinated but plants after subculturing failed to grow further. Aseptic germination was prolonged with intention to obtain callus. After two months a culture was observed in which the seed proliferated callus from which a single shoot differentiated and continued to grow after subculturing. From this explant a clone of shoot cultures was established.

BA and IBA concentration were varied until medium supplemented with 0.1 mgl^{-1} of both BA and IBA was chosen as optimal. Growth of shoot cultures was not as fast as callus proliferation. Optimal source of explants for shoot multiplication were

6-12 mm long shoots joint into shoot clusters, Fig. 1. Individual, elongated shoots were not suitable for shoot multiplication. Stocks with shoot cultures of *P. hirsuta* were maintained continuously for four years.

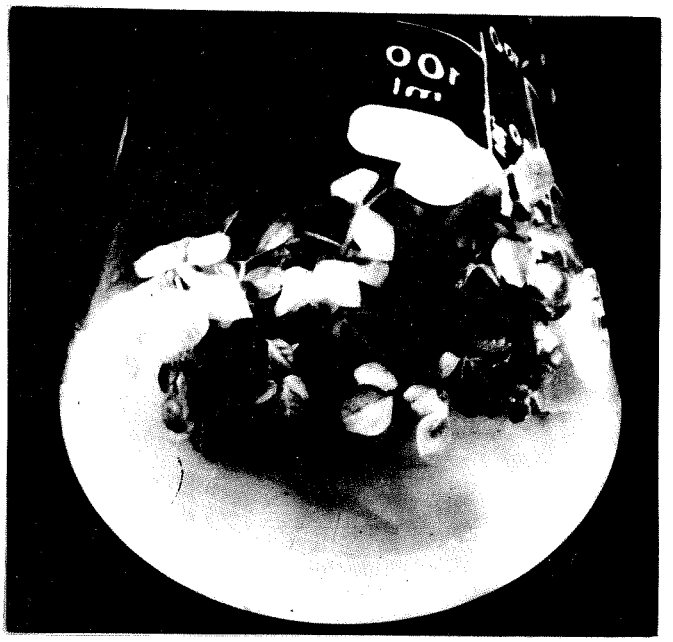

Fig. 1. – Shoot clusters comprising large and small shoots with compound leaves of various size

Rooting was performed on medium containing 0.1 mgl^{-1} IBA or on hormone free medium. Roots were long and slender. Plants were sensitive and difficult to adapt. They had a strong tendency to loose leaves upon transfer *ex vitro*, requiring a rest period to resume growth and form new leaves.

Perhaps the most important finding was that the complex leaf structure was unhindered by *in vitro* conditions. This means that leaves were always compounds, three independent leaflets could be observed even on very small leaves. In other plant species with compound leaves often a reduction in number of leaflets occur as for instance in carob. In this species usually only the first pair of leaflets develop (Vinterhalter & Vinterhalter, 1994). True, compound leaves in carob develop later after adaption.

During *in vitro* culture leaves were indifferent to light. The ability to track the light source developed only after the successful adaptation of plantlets to *ex vitro* conditions (Fig. 2). However, it is interesting to note that leaves formed *in vitro* were morphologicaly complete and that reasons for which leaves fail to perform sun tracking *in vitro* need to be investigated further.

Fig. 2. – Addapted plantlets, arangement of leaflets in the same plane – beginning of suntracking movements

Although shoot cultures of *P. hirsuta* were obtained and maintained for four years in our laboratory, we still consider this species as recalcitrant for *in vitro* propagation. It was difficult to find explants from which shoot cultures could be established. There were also problems which accompanied adaptation and further growth of adapted plants.

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Rezime

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Pueraria hirsuta (Thunb.) se u botaničkoj bašti „Jevremovac" u Beogradu gaji kao retka tropska vrsta koja u odsustvu odgovarajućih oprašivača ne obrazuje mahune sa klijavim semenom. Obzirom da se vegetativno razmnožavanje vrši samo putem položnica to je istražen postupak za *in vitro* razmnožavanje ove vrste. Različiti tipovi eksplantata uključujući bočne pupoljke, segmente izdanaka i pulvinusa su kultivisani na modifikovanoj MS (Murashige i Skoog, 1962) podlozi u koju su dodati 0-1.0 mgl^{-1} BAP i NAA. Aktiviranje i rast bočnih izdnaka nisu dobijeni, eksplantati su obrazovali samo kalus iz kojeg se izdanci nisu mogli diferencirati. Najveća proliferacija kalusa bila je na podlogama sa 0.2 mgl^{-1} BAP i NAA odnosno na podlozi sa 1.0 mgl^{-1} BAP i 2.0 mgl^{-1} NAA. Oko 200 semenki prikupljeno je sa jednog primerka biljke u blizini Dubrovnika od čega je 60 najkrupnijih izdvojeno i postavljeno na aseptično isklijavanje na MS podlogu sa 0.5 mgl^{-1} BAP i 0.1 mgl^{-1} IBA. Samo dve semenke su proklijale, ali su obe biljke propale nakon pasažiranja. Nakon dva meseca još jedna semenka iz ove grupe je reagovala i produkovala kalus iz kojeg se zatim diferencirao jedan izdanak. Ovaj izdanak je uspešno pasažiran i od njega je formiran klon kultura izdanka koji je zatim uspešno održavan 4 godine na podlozi sa 0.1 mgl^{-1} BAP i NAA. Izdanci ožiljeni na podlozi bez hormona su uspešno adaptirani u staklari.