Proceedings of the 5th Balkan Scientific Conference on Biology

Kostadinova, S., Mollov, I., Dzhambazov, B., Naimov, S., Vassilev, K. & Georgiev, B. (Eds.)
Plovdiv, Bulgaria ● 15-16 April 2021 ● pp. 111-117

BalkanBio

Overcoming Phytoplasma Infection in Paulownia tomentosa by Meristem In vitro Culture

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Abstract. Paulownia witches' broom (PaWB) is caused by phytoplasma of Aster Yellows group and may result in severe damage to the growth and premature death of the infected tree. *In vitro* cultures of meristems are important tools for solving problems with plant diseases. In the present study, meristem cultures from phytoplasma infected *Paulownia tomentosa* have been created in an attempt to obtain healthy plants. The faster procedure with longer explants generated 95% regeneration but no healthy plants. The combination of smaller explants and thermotherapy resulted into the generation of plantlets with a healthy phenotype. The PCR analyses showed the absence of phytoplasma only in one of the produced plants. Despite the low efficiency, expressed in one phytoplasma-free regenerant of a total of 480 explants, these results can serve to continue the work on optimizing the used strategy.

Key words: meristem plant tissue culture, thermotherapy, Paulownia witches' broom (PaWB).

Introduction

Paulownia tomentosa (royal paulownia, empress, or princess tree) is a deciduous and fast-growing tree of the family Paulowniaceae. It has several desirable characteristics and nowadays is grown in many countries including China, U.S.A., west and south Europe (Newman et al., 1998; Ipekci & Gozukirmizi, 2004). It is not only an ornamental tree but also multipurpose species with potential medicinal uses, a source of renewable energy and also used for reforestation and reclamation of mine sites (Rao et al., 1996; Ozaslan et al., 2005; Barton et al., 2007). Paulownia trees have been cultivated for their commercial importance but they are susceptible to Paulownia witches' broom (PaWB), which is one of the most devastating diseases caused by phytoplasma of the Aster Yellow group "*Candidatus Phytoplasma asteri*" (Doi et al., 1967). Aster yellows (AY) group (16SrI) phytoplasmas are associated with over 100 economically important diseases worldwide and represent the most diverse and widespread phytoplasma group. Phytoplasmas are cell wall-less plant pathogenic bacteria that belong to the class Mollicutes and have unique biology among bacteria. These obligate intracellular parasites need plants and insects for survival in nature and they can efficiently reproduce in both hosts (Hogenhout, 2009). The geographical distribution and impact of phytoplasma diseases depend on the host range of the phytoplasma as well as on the presence and the feeding behaviour of the insect vector. Characteristic symptoms associated with phytoplasma infection include witches' brooms (proliferation of auxiliary or axillary shoots), short internodes, yellowing or reddening of leaves, phyllody (the development of floral parts into leafy structures), stunting and decline, virescence (the development of green flowers and the loss of normal flower pigments), sterile flowers and necrosis (Khadhair et al., 2001; Namba, 2002; Bayliss et al., 2005; Fan et al., 2015). Plants infected by members of the 16SrI subgroups include general

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stunting (little leaves, small flowers, shortening of internodes), leaf curl or rolling, small and faintly coloured flowers, and some symptoms that are typical of the AY syndrome (Lee et al., 2004).

Studies have been carried out to understand the interaction of paulownia with phytoplasma but the molecular mechanism of PaWB occurrence remains elusive (Liu et al., 2013; Cao, 2014).

In vitro culture techniques represent successful strategies for the production of pathogens-free plants (Panattoni et al., 2013; Smith et al., 2017). Meristem tip culture is extensively used for eradication of plant pathogens, which involves the removal and *in vitro* placement of dome-shaped meristematic tissue on the culture media for the development of the whole plant. This technique has been used alone or in combination with other treatments for virus elimination from plants. Meristem tip culture of 0.3 mm has been useful to eliminate the virus in sweet potato and cassava. However, meristem tips of larger size (1-2.5 mm) have also given satisfactory results in virus elimination when heat treatments are provided (Nehra & Kartha, 1994; Varveri et al., 2015).

The aim of the present study was to regenerate healthy plants from PaWB-infected *Paulownia tomentosa* using the meristem cultures technique. Plant regeneration has been achieved from excised tissues with variation in their sizes and combined with thermotherapy and callus tissue.

Material and Methods

Plant materials. PaWB-infected plants were cultivated on MS media (Murashige & Skoog, 1962) containing 30 g/L sucrose and 7 g/L plant agar. The pH of the media was adjusted to 5.8 before autoclaving. The culturing conditions were 16-h photoperiod with cool-white fluorescent tubular lamps at 25±2 °C.

Meristem plant tissue culture. Under a laminar flow hood, meristem tips were removed by sterile dissection under microscope from apical shoots and axillary buds of infected plants. The explant comprises the apical dome and a limited number of the youngest leaf primordia and has the potential for excluding pathogenic organisms that have been present in the donor plants. The explant size plays a major role in meristem culture and we tested variants. The first pattern for meristem culture was with tips 1-2.5 mm in length, which made the experiment faster and easier, compared to the second variant where the meristem tips were with smaller size (0.5 mm). In the third variant, we used explants 0.5 mm in length but donor plants were preliminarily cultivated for one week at 40 °C (thermotherapy). Tips from the described three experimental patterns were cultured in Petri dishes, each with 20 mL of initiation MS media containing 0.2 mg/L NAA (α-naphthalene acetic acid) and 0.5 mg/L GA_3 (gibberellic acid) (20 tips per Petri in two repeats). Each experiment was performed in triplicate. The samples were cultured at 25±2 °C in the dark for two weeks. Thereafter, they were transferred at a light with 16/8 h (light/dark) photoperiod for another week. Twenty-one days after the beginning of initiation, produced shoots were excised and transferred to elongation MS media containing 1 mg/L BAP, 0.1 mg/L NAA, and 1 mg/L GA_3. Four weeks after the beginning of elongation, growing seedlings were observed for morphological changes.

In the fourth pattern, a combination of callus and meristem tissue culture was tested, where explants (1-2.5 mm in length) were introduced to MS media with 5 mg/L BAP (6-benzylaminopurine) and 0.5 mg/L GA_3. Two weeks explants were in the dark at 25±2 °C, thereafter Petri dishes were moved to cool-white fluorescent tubular lamps light (16/8 photoperiod) for two more weeks. The formed callus was transferred to MS media with 1 mg/L BAP and 0.1 mg/L NAA for shoot induction. Every four weeks callus tissue was transferred to fresh media. Excised shoots were grown on MS media without plant growth regulators.

The newly formed shoots with healthy phenotype were tested for PaWB detection through PCR analysis.

DNA extraction. Total DNAs were extracted from analysed shoots with innuPREP Plant DNA kit (Analytik Jena AG, Jena, Germany) following manufacturer's instructions. The quality and concentration of isolated DNA were estimated on 1% agarose gel and spectrophotometric analysis.

PaWB phytoplasma detection. In direct PCR assays, amplification of phytoplasma 16S rDNA was performed by using R16F2n (GAAACGACTGCTAAGACTGG) and R16R2 (TGACGGGCGGTGTGTACAAACCCCG) universal phytoplasma primers to amplify DNA fragments of 1.24 kb. Each reaction was performed and PCR products were analysed as previously described by Lee et al. (1993). DNA products from PCR assays were separated in 1% agarose gels and compared with a standard molecular marker (2-log DNA Ladder 0.1-10kb, New England Biolabs, Ipswich, Massachusetts, USA).

Results

Pattern 1. Meristem tissue culture from explants 1-2.5 mm in length.

This experimental pattern presented total of 114 plantlets arisen from 120 explants, used in the tree repeats, or av. 38 plants per 40 excised tips (Table 1), and none of the newly formed shoots had healthy phenotype (Fig. 1A).

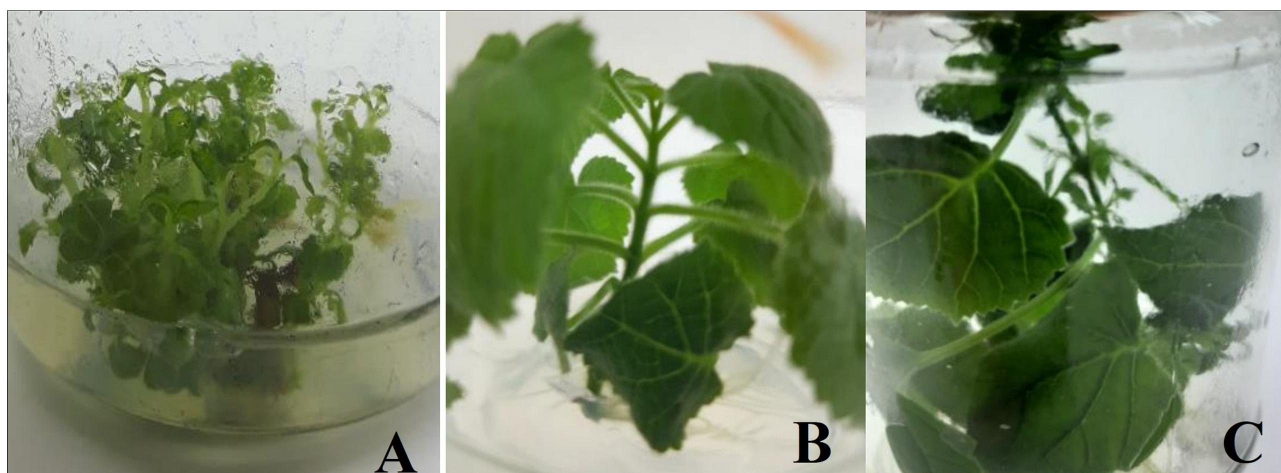

Fig. 1. Changes of morphology in *Paulownia tomentosa* plantlets: (A) with PaWB infection; (B) after thermotherapy and phytoplasma eradication; (C) after indirect regeneration from callus tissue that resulted in bigger leaves and enlarged internodes like healthy phenotype but with proliferation of axillary shoots that is witches' broom symptom.

Pattern 2. Meristem tissue culture from explants 0.5 mm in length.

The excised smaller explants have revealed lower regeneration efficiency (Fig. 2), with only 54 regenerants from a total of 120 used meristem tips (Table 1). In this variant, despite the smaller sizes of the selected tissues, all newly produced plantlets had no changes in their morphology, repeating PaWB symptoms – small light green to yellow leaves and short internodes with sprouting axillary buds.

Pattern 3. Thermotherapy and meristem tips – 0.5 mm in length.

When we combined smaller explants with thermotherapy, less than 40% of excised meristem tips regenerated to plantlets, presenting a decrease in regeneration efficiency, compared to the first and second experimental patterns (Fig. 2). On the other hand, changes in phenotype appeared and

three of the grown shoots regained a healthy morphology – bigger and depth green leaves, enlarged internodes, without developing axillary buds (Fig. 1B).

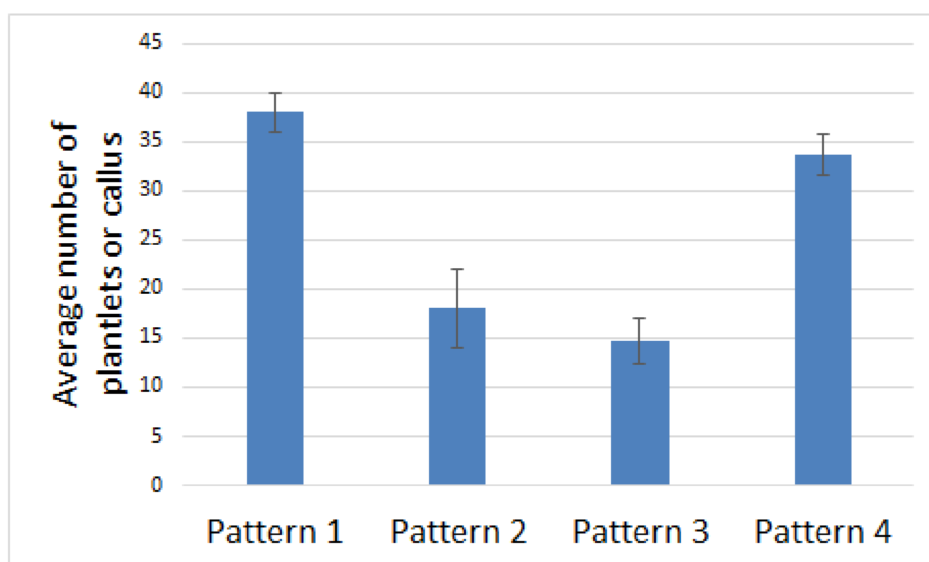

Fig. 2. Average number of plantlets (callus for pattern 4) in the applied experimental designs.

In the followed PCR analysis with universal R16F2/R2 primer pair, from the first newly formed plant with a healthy phenotype (3.1) produced through thermotherapy and 0.5 mm meristem-tips, there was no amplified phytoplasma 16S rDNA. However, 1.2 kb long products were amplified from 3.2 and 3.3 (Fig. 3). The results showed that tree promising samples were produced out of a total of 120 explants in this experimental pattern but only one was free from phytoplasma, with 0.83% efficiency in overcoming witches' broom infection.

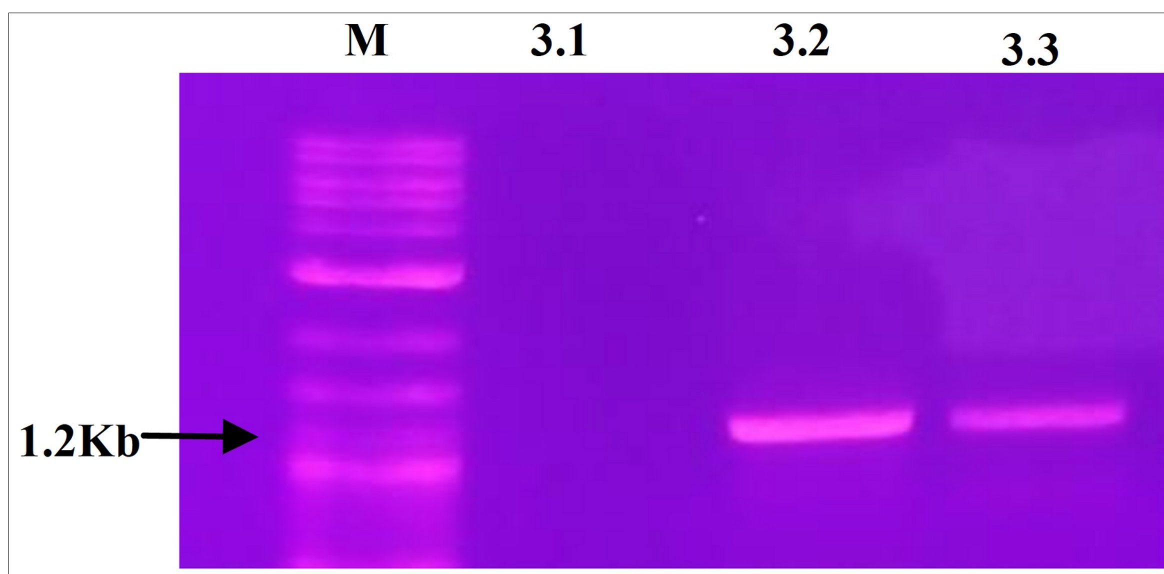

Fig. 3. Detection of phytoplasma 16SrDNA by PCR. **M:** DNA Marker; **1**: (3.1) the first regenerant with healthy phenotype in experimental pattern 3; **2:** (3.2) the second regenerant with healthy phenotype; **3:** (3.3) the third regenerant with healthy phenotype.

Pattern 4. Combination of callus and meristem culture with explants 1-2.5 mm in length. Most of the excised tips, for this experimental design, developed to callus tissue (Table 1). The induced calli were placed on a fresh medium, where they produced more than one shoots. Many of the excised shoots were asymptomatic (35.83%) but the healing effect was not permanent. In the prolonged cultivation, PaWB phenotype reappeared (Fig. 1C). This pattern was repeated three times with 214 shoots generated in total. After three months cultivation, one of the regenerated plants kept a healthy phenotype. To investigate the presence of pathogens' DNA, the plant that kept the changed morphology was tested with PCR. Through the analysis 1.2 kb fragments were amplified, revealing lack of success in overcoming phytoplasma infection (Fig. 4).

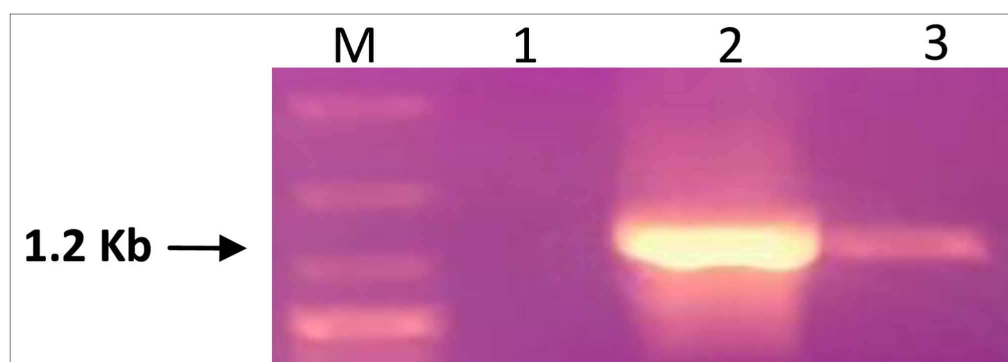

Fig. 4. Detection of phytoplasma 16SrDNA. **M:** DNA Marker; **1:** - Control (distilled water); **2:** +Control (PaWB infected plant); **3:** Regenerant with healthy phenotype.

Table 1. Efficiency of patterns. The regeneration efficiency in the fourth pattern was calculated about frequency of callus induction. Each experiment was repeated tree times.

Experimental pattern	Number of explants per experiment	Average number of plantlets or callus*	Regeneration efficiency	Total number of plants with changed phenotype	Total number of plants free from phytoplasma
Pattern 1	40	38±2	95%	0	0
Pattern 2	40	18±4	45%	0	0
Pattern 3	40	14.7±2.309	36.7%	3	1
*Pattern 4	40	33.7±2.081	84.17%	43	0

Discussion

In order to select pathogens free cells, the smallest possible meristem has to be taken but small explants could not survive because of desiccation, while large meristems do. After conducting four different strategies for meristem cultures, it has been observed the disappearance of PaWB symptoms in 46 produced regenerants of a total of 480 used explants. Mostly, the combination of meristem tissue with indirect regeneration through callus tissue reduced phytoplasma' symptoms (35.8%), compared to the variant with thermotherapy (2.5%), and lack of changes in the other two patterns. The healing effect turned to be permanent for one of the changed plantlets, and it was the product of a combination of smaller explants and thermotherapy.

As it is already known, the size of shoot tips is critical for pathogen eradication but it is also positively related to survival and shoot regeneration (Wang et al., 2018). In our studies, shoot regeneration levels were much higher (95%) in meristem culture from larger explants (1-2.5 mm) than that (45%) in meristem culture from tips 0.5 mm in length. When heat-treatment has been applied the regenerative ability of excised 0.5 mm tips reduced to 36.7%. Heat therapy is the oldest method used for the elimination of viruses, viroids and phytoplasmas from vegetatively propagated plants and has been used since the end of the nineteenth century (Varveri et al., 2015). High temperatures induce stress to plants and such stress is intensified as their durations increase (Wahid et al., 2007). Negative effects of prolonged thermotherapy durations on *in vitro* shoots and tips excised from the treated shoots were found in woody plants such as apricot, peach and cherry (Gella & Errea, 1998). In this study, PaWB-infected *in vitro* plants were heat-treated for one week at 40 ˚C and then used for meristem-tip cultures, which produced tree symptomless shoots and one of them was proved to be free from phytoplasma. Despite the low efficiency of the tested strategies, the provided data can serve to continue the study on the eradication of phytoplasma infection in paulownia plants. It has been suggested that the indirectly regenerated plants with changed morphology could be appropriate donor plats for further experiments with meristem tips for pathogen eradication.

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