Scientific Papers. Series D. Animal Science. Vol. LXVII, No. 1, 2024
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

RESEARCH ON THE USE OF MULBERRY VITROPLANTS IN ORDER TO PRACTICE A SUSTAINABLE AGRICULTURE

Georgeta DINIȚĂ[1], Mihaela HĂBEANU[2], Cristiana DIACONESCU[1], Anca GHEORGHE[2], Teodor MIHALCEA[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[2]Research Station for Sericulture Băneasa, 013685, Bucharest, Romania

Corresponding author email: mihaela.habeanu@scsbaneasa.ro

Abstract

The conservation of sericultural vegetative genetic resources is a permanent concern in the sericulture field, being known that the mulberry leaf is the only source of food for the silkworm Bombyx mori L. These sericultural vegetal genetic resources are mainly used to obtain planting material used for establishment the mulberry plantations, but also for the mulberry improvement program and the establishing of the phytoremediation potential of mulberry plants. The research carried out for the preliminary testing in laboratory of in vitro multiplication potential of some mulberry varieties have highlighted the possibility of using the biotechnologies of tissue cultures, meristems and organs for testing the phytoremediation potential of mulberry plants with sericultural destination. Thus, a planting material was produced, consisting of mulberry vitroplants colonized with vesicular-arbuscular endomycorrhizae, which keep the genetic characteristics of parental forms, but present superior bioproductive parameters and physiological tolerance on soils contaminated with lead.

Key words*: mulberry, silkworm, vesicular-arbuscular mycorrhizae.*

INTRODUCTION

Land degradation processes by human activities are very varied, sometimes producing the soil damage or even the total anihilation of its functions into ecosystems (Jie et al., 2002; Nkonya et al., 2016; Sutton et al., 2016; Zhang et al., 2023).

Due to soil contamination, the quality of the harvest is depreciated and the agricultural production is reduced or compromised, the consequences being felt in the entire chain: soil - micro-organisms - plants - animals - human (Dotaniya et al., 2020; Angon et al., 2024).

The cause of the heavy metals accumulation in soil may be of *geogenic* nature (as a result of the geochemical processes of rock and minerals alteration) or of *anthropogenic* nature (as a result of human activities: fertilizers, amendments, pesticides, gases or dusts from the atmosphere from various industries or from combustions) (Haque et al., 2009; Pečiulyté et al., 2009; Kumar et al., 2015). The degradation of agricultural land also takes place through processes of contamination/pollution with heavy metals: iron, manganese, copper, zinc, lead, cadmium, chromium, cobalt, nickel (Alengebawy et al., 2021; Rashid et al., 2023).

The research and development in the field of technologies of physical, chemical and biological remediation of soil pollution with heavy metals have evolved significantly, facilitating their currently use in order to counter this major environment problem (Fan et al., 1013; Lan et al., 2020; Srivastava et al., 2022).

Adopting the sustainable agricultural practices is important for the conservation of sericultural vegetative genetic resources considering that the mulberry leaf (*Morus* spp.) is the only source of food for the silkworm *Bombyx mori* L., but also for maintaining the ecological balance in ecosystems with mulberry plantations (Rohela et al., 2020; Hăbeanu et al., 2023).

The processes of bioremediation in situ are important for pollution control, the study and implementation of such processes being able to be conceived by investigations on natural mechanisms of absorption, biotransformation, bioaccumulation and toxicity of pollutants in plants and micro-organisms (Liu et al., 2018; Diaconu et al., 2020).

The silkworms having a high sensitivity of the organism can be used as bio-indicator to detect the environment pollution. Thus, the high content of heavy metals influences the development of biological characteristics, mostly the silk filament length and weight (Nikolova, 2019).

One of the main research directions in the sericultural field consists in the reducing of the dependence on chemical fertilizers through the implementation of ecological agricultural practices (Vinod et al., 2020; Chakraborty et al., 2016; Saqib et al., 2024).

The phytoremediation is recognized as being an ecological and cost-effective approach to remediate the risks of soil pollution (Lan et al., 2020; Xianghong He, 2023; Deng et al., 2024).

Considering that the pollution produces un-balance in the mycorrhizae associations it can be proceed at phytoremediation of land contaminated by using plants from *Morus* genus (Baqual et al., 2017; Ma et al., 2022).

Among these practices stands out the biofertilizers using, such as vesicular-arbuscular mycorrhizae (VAM), in the form of commercial products obtained by biotechnology, together with extraradicular fertilizing (Rajaram et al., 2014; Berruti et al., 2016; Diniță et al., 2023). This approach promotes greater sustainability in the sericultural production, contributing to the environment protection and to the maintaining of ecological balance of agricultural ecosystems (George et al., 2023; Sun et al., 2023).

The obtaining of planting material from the valuable mulberry varieties is made exclusively by vegetative means, by usual methods (cutting, marking, grafting) or by *in vitro* regeneration biotechnologies (Gecer et al., 2016, Choudhary et al., 2023). In the sericultural field the conservation of vegetal genetic is also achieved by utilizing the vegetative cloning, due to the fact that the mulberry being a monoecious or dioecious plant with unisex flowers, it presents a pronounced heterozygous character of the descendants (Tikader et al., 2009).

The *in vitro* cloning consists the only vegetative multiplying method of the mulberry varieties reluctant to cutting and marking, the graft being used to obtain mulberry varieties destined for establishing mulberry plantations with forms of tall bushes, half-trunk and tall trunk, because the production cuttings consist in completely cutting the annual herbaceous shoots (Taha et al., 2020). The biotechnologies of mulberry plants multiplication refer to vitroplants obtaining by tissues culture, meristems and by cells culture (Vijayan et al., 2014; Saha et al., 2016; Litwińnczuk et al., 2020).

The using of *in vitro* multiplying biotechnologies offers the possibility to reduce the time necessary to highlight the accumulation of different substances in various plants' organs (Choudhary et al., 2015).

The researches aimed to apply the work protocol elaborated to obtain mulberry vitroplants and to establish the optimum conditions for *in vitro* multiplication of some valuable genotypes, located in the collection of mulberry varieties in Research Station for Sericulture Baneasa Bucharest.

To establish the phytoremediation potential of the mulberry plants, the researches aimed to highlight the impact of lead contamination of mulberry plants in the biological processes of their germination and development.

MATERIALS AND METHODS

The vegetative propagation *in vitro* is based on the meristems' formation and multiplication, which represents the tissues with the greatest genetic stability. The *in vitro* micro-propagation can have as starting point the pre-existent meristems (the embryonic apex, the apex of the main shoot or the axillary ones), or adventive meristems, induced by the technique of *in vitro* culture.

The design of an experimental *in vitro* model to obtain mulberry microplants was made taking into account the type of explants used, respectively herbaceous shoots developed in laboratory conditions by dormant buds' forcing from the branches of the mature mulberries, belonging to genotypes resistant to cutting – China 98 and China 99 (*Morus multicaulis*) and to mulberry plants obtained from seeds (hybrids) in aseptic conditions.

The work protocol includes the following steps:

- *obtaining the source of explants* is made by forcing in laboratory conditions the lignified shoots harvested in the period November – December from the mulberry plantation (vegetative rest period). The forcing is done by simulating budding conditions, placing these

shoots in vessels with tap water and ensuring an ambient temperature of 24-25°C. After 60 days, from the dormant buds will develop apically the herbaceous shoots, which will constitute the source of explants;

- *the sterilizing of the vegetal material* consists in an operation of washing with normal tap water (pre-sterilizing) necessary to remove mechanically some contaminants. The herbaceous shoots are cut in pieces of about 1 cm, each containing one axillary bud, this becoming a nodal explant. The effective sterilization was made with $HgCl_2$ in concentration of 0.03% for 30 minutes, the nodal explants being continuously mixed in this solution, after which they have been washed with distilled water for 10 minutes of three rounds. The sterilization can also be done using a solution of 3-5% with potassium hypochlorite;

- *the initiation of primary culture (inoculation)* takes place in aseptic conditions at the laminated flow hood. The culture environment used is of Murashige-Skoog type with 0.8% agar distributed in culture vessels of "baby food" type, the culture medium being supplemented with the following hormone variants:

V1 – 1 mg/l BAP (Benzylaminopurine)
V2 – 1 mg/l K (Kinetin)
V3 – 2 mg/l BAP
V4 – 2 mg/l K
V5 – 1 mg/l BAP + 1 mg/l K
V6 – 2 mg/l BAP + 2 mg/l K
V7 – 2 mg/l BAP + 1 mg/l K
V8 – 1mg/l BAP + 2 mg/l K

After inoculation (placing the nodal explants in culture vessels sterilized on the culture medium with respect of normal polarity) the culture vessels are covered with parafilm;

- *the incubation of initial culture* – the multiplication consists in transferring into the incubation room (for growing) of the culture vessels for 15 days at the temperature of 25-28°C and with artificial lighting, with a photoperiod of 16:8 hours at a 3,000 lux intensity;

- *subculturing* is necessary to obtain clones from the same apex in the conditions of caulinar apexes using, by transferring from the initial culture of some explants in culture vessels with fresh medium;

- *rooting and regeneration of autonomous plants* is made when the shoots developed in the culture vessels reach the length of 1.5-2 cm and has as its purpose the rootlets obtaining by changing the culture medium, where the formula remains unchanged, except the agar which is no longer added, the culture medium being liquid. The maintaining of vitroplants in the liquid culture medium is made by utilizing some bridges of filter paper, the rooting being ensured by supplementing the culture medium with IBA (Indole-3-Butyric-Acid) hormone in concentration of 1 mg/l. The environment conditions of temperature and light are kept at the same parameters. The first root primordia appear at an interval of 10-15 days after passing on the liquid culture medium;

- acclimatization is the stage in which the plants are transferred in culture vessels, after they have been well washed beforehand from the remains of the culture medium. For the *in vitro* multiplied plants to be transferred in the culture medium itself, it is necessary the gradual adaptation of the root and leaf system of plants. In this acclimatization stage was made the inoculation with vesicular-arbuscular mycorrhizae of the vitroplants utilizing the product Endorize SOL in dose of 15 mg/plant.

During the researches there were carried out laboratory tests concerning *in vitro* multiplication of some mulberry varieties with organogenetic regeneration potential.

To highlight the impact of Pb contamination of mulberry plants, the planting material used was obtained from mulberry seed, the mulberry plants being unisexual dioecious from the botanic point of view and heterozygous, hybrids in first generation, from genetic point of view.

The experiment was carried out in *ex situ* conditions.

The experiment concerning the lead contaminants impact on the germination process of the mulberry seeds used the following work variants:

- V_0 Control represented by 10 mulberry seeds germinated on filter paper with tap water in Petri vessels;

-V_1 Variant represented by 10 mulberry seeds germinated on filter paper with solution of Pb $(NO_3)_2$ in concentration of 0.4 mg Pb% ml solution;

-V_2 Variant represented by 10 mulberry seeds germinated on filter paper with solution of Pb

$(NO_3)_2$ in concentration of 0.04 mg Pb% ml solution.
Each variant was made in two repetitions, the solutions being completed how many times their level was decreasing. At an interval of 7 days from the date of starting the simulation, the first observations were made concerning the seed swelling, the hypocotyl appearance, its elongation, the photosynthesis appearance by coloring the hypocotyl green.
The experiment concerning the lead contaminants impact on the replanting process of the mulberry plants was made in the conditions of utilizing some identical concentrations mentioned in the germination case (water and Pb $(NO_3)_2$). The mulberry microplants have been obtained by sowing a quantity of 30 g mulberry seeds in a substrate consisting of flower soil sterilized in oven at 105°C for 8 hours. Then, the obtained plants have been replanted into a culture vessel with the same substrate, when their high was of 10 cm minimum and at which the lignification process at the parcel level was noticed.
The observations concerning the lead contamination of the substrate were made weekly until the end of the work stage.

RESULTS AND DISCUSSIONS

Results concerning the *in vitro* multiplication potential of some mulberry varieties

The research concerning the *in vitro* multiplication of some mulberry varieties highlighted their organogenetic regeneration potential.
Regarding the morphogenetic capacity of the sampled apexes from the mulberry varieties taken into study, it was found that the meristematic apexes aseptic inoculated on the eight variants of experimented nutritive mediums have developed shoots in variable number, in accordance with the genotype and the hormonal supplement administrated (Tables 1, 2, 3). It is worth noting the accentuation of morphogenetic capacity in time, after 3-4 subculturing, under the effect of some balanced combinations of BAP (Benzylaminopurine) and Kinetin when there have been developed multiple shoots from every apex. The varieties China 98 and China 99 and the mulberry hybrids come from seed can be multiplied by *in vitro* cloning biotechnology in compliance with the work protocol which utilizes 2 mg/l BAP + 1 mg/l K or 1 mg/l BAP + 2 mg/l K, that determined maximum values for the length of the shoots developed from meristematic apexes.
The research on inducing rootedness and regeneration of autonomous plants aimed the effect of auxins IBA (Indole-3-butyric-acid) and 2.4-D (Dichlorophenoxyacetic acid) on the developing of adventitious roots at the base of shoots elongated at 2-5 cm, in the morphogenetic cultures of *Morus* sp. genotypes studied. The rooting of vitroplants presented maximum values of 70-100% in the contidions of utilizing IBA (Indole-3-Butyric-Acid) - 1 mg/l and 2.4-D (Dichlorophenoxyacetic acid) - 1 mg/l (Table 4).
The experimental data concerning the mycorrhizae influence on the mulberry microplants obtained by *in vitro* cultures from the varieties China 98, China 99 and mulberry hybrids highlight greater values for this biofertilization. The mulberry hybrids Eforie presented a seedling length of 65.04 cm and a number of 24 leaves/seedling in the case of mycorrhizae utilization, while in the variant of chemical fertilization NPK, they had a number of 20 leaves/seedling with a seedling length of 64.28 cm (Table 5).

Results concerning the testing of phytoremediation potential of mulberry plants with sericulture destination

The biological processes studied in conditions of lead contamination have been represented by seed germination and plants replanting.
The experimental results concerning the lead contaminants impact on the germination process of mulberry seeds highlighted the following aspects:
- the process of mulberry seeds swelling was observed to all work variants;
- the process of hypocotyl appearance and development was observed in variants and V_0 and V_2;
- the process of hypocotyl appearance is blocked in the case of variant V_1, that constitutes the maximum limit of lead contamination of the germination substrate against which is considered the tolerance to germination of the mulberry seeds;

- the process of photosynthesis appearance is observed in both variants mentioned before, but the development pace is higher in variant V_0.

The results concerning the viability of mulberry seeds in the conditions of simulating lead contamination are presented in Table 6.

In *ex situ* conditions, the accumulation of lead contaminants in mulberry plants influences the germination process under its blocking aspect in concentrations higher than 0.4 mg Pb% ml solution, the nitrate inhibiting the enzymatic biochemical processes. The concentration interval 0.0-0.04 mg Pb% ml solution doesn't influence the germination, the mulberry leaves being tolerant to lead contaminants in the form of salts, respectively lead nitrate.

In the conditions of *ex situ* lead contamination of the transplanted seedlings, the results of this biological testing highlight the fact that the transplanted mulberry plants do not present visible phenotypic modification in V_0 and V_2, the tolerance interval remaining the same both in the germination process and in the development process of the mulberry plants, including the radicular system.

In the two variants do not exist significant differences concerning the high of the transplanted seedlings (Table 7).

Table 1. The effect of the hormonal supplement on the shoots developing in *in vitro* conditions - mulberry variety China 98

Experimental variant	Apexes started growing (%)	Medium number of shoots/explant	Medium length of shoots (cm)
1 - 1 mg/l BAP*	50	2	1.7
2 - 1 mg/l K**	30	3	2.2
3 - 2 mg/l BAP	75	3	2.5
4 - 2 mg/l K	45	2	1.8
5 - 1 mg/l BAP + 1 mg/l K	85	3	2.7
6 - 2 mg/l BAP + 2 mg/l K	70	3	2.5
7 - 2 mg/l BAP + 1 mg/l K	100	4	3.7
8 - 1 mg/l BAP + 2 mg/l K	100	3.5	3.1

*Benzylaminopurine; **Kinetin

Table 2. The effect of the hormonal supplement on the shoots developing in *in* vitro conditions - mulberry variety China 99

Experimental variant	Apexes started growing (%)	Medium number of shoots/explant	Medium length of shoots (cm)
1 - 1 mg/l BAP*	50	2	1.5
2 - 1 mg/l K**	35	2.5	2.4
3 - 2 mg/l BAP	80	2.5	2.3
4 - 2 mg/l K	50	2	1.9
5 - 1 mg/l BAP + 1 mg/l K	90	3	2.5
6 - 2 mg/l BAP + 2 mg/l K	75	3.5	2.3
7 - 2 mg/l BAP + 1 mg/l K	100	4.5	2.8
8 - 1 mg/l BAP + 2 mg/l K	100	4	2.5

*Benzylaminopurine; **Kinetin

Table 3. The effect of the hormonal supplement on the shoots developing in *in vitro* conditions - mulberry hybrids

Experimental variant	Apexes started growing (%)	Medium number of shoots/explant
1 - 1 mg/l BAP*	50	6
2 - 1 mg/l K**	35	7
3 - 2 mg/l BAP	80	8
4 - 2 mg/l K	50	8
5 - 1 mg/l BAP + 1 mg/l K	90	9
6 - 2 mg/l BAP + 2 mg/l K	75	10
7 - 2 mg/l BAP + 1 mg/l K	100	11
8 - 1 mg/l BAP + 2 mg/l K	100	15

*Benzylaminopurine; **Kinetin

Table 4. The percent of rooted shoots depending on the type and concentration of auxin

Type and concentration of auxin (mg/l)	Mulberry genotype – shoots rooting (%)					
	Hybrids Kokuso 21	Hybrids Ichinose	Hybrids Eforie	Hybrids Olteni	China 98	China 99
IBA - 1 mg/l	70	75	85	98	80	80
2.4-D - 1 mg/l	60	70	80	100	70	75

Table 5. The mycorrhizae influence on the growing of mulberry microplants

Somatometric character	Fertilization type	Mulberry genotype			
		China 98	China 99	Hybrids Kokuso 21	Hybrids Eforie
Seedlings length (cm)	Mycorrhizae	29.12	29.40	64.44	65.04
	NPK	27.64	19.00	64.68	64.28
Number of leaves	Mycorrhizae	22	16	14	24
	NPK	21	12	13	20

Table 6. The viability of seeds in conditions of lead contamination

Variant	Concentration (mg Pb % ml solution)	Viability (%)
V_0 Uncontaminated control	0	100
V_1 Lead contamination	0.4	0
V_2 Lead contamination	0.04	50

Table 7. The height variation of transplanted seedlings with the lead concentration applied to the germination substrate

Variant	Concentration (mg Pb % ml solution)	Viability (%)
V_0 Uncontaminated control	0	22.0
V_1 Lead contamination	0.4	0
V_2 Lead contamination	0.04	19.8

Figure 1. Changes concerning the appearance and consistency of mulberry leaves in the case of variant V_1 (0.4 mg Pb% ml lead nitrate) (Own source)

The presented data highlight the interval of tolerance and growing of the mulberry plants in the value margin of 0,0-0.04 mg Pb% solution. The transplanted seedlings in the frame of V_1 variant presented after a time interval of 15 days, the first signs of changes in the leaves aspect and consistency by the appearance of some insular discolorations similar to mycosis attack, the basal leaves drying up completely and the apical growth leaves twisting and having an embossed appearance (Figure 1). Until the end of the stage, the mulberry plants are still viable, but with strong appearance changes and with unfavorable prognosis.

As a result, in the conditions of *ex situ* lead contamination of the transplanted mulberry seedlings, they presented tolerance to the pollutant up to the concentration of 0.04% and at the concentration of 0.4% the lead contamination of the transplanted mulberry plants they present visible phenotypic changes with unfavorable prognosis.

CONCLUSIONS

The preliminary laboratory testing of the *in vitro* multiplication potential of some mulberry varieties highlighted the possibility of utilizing biotechnologies of tissues, meristems and organs cultures in order to test the phytoremediation potential of mulberry plants.

In *ex situ* conditions the accumulation of lead contaminants in mulberry plants influences the germination process of its blocking aspect in concentrations higher than 0.4 mg Pb/100 ml solution. The concentration interval 0.0-0.04 mg Pb% ml solution does not influence the germination, the mulberry plants being tolerant to lead contaminant in the form of salts, respectively lead nitrate.

In the conditions of *ex situ* lead contamination of transplanted mulberry seedlings, they manifest tolerance to pollutant up to the concentration of 0.04 mg Pb % ml solution, the developing process being carried out without significant differences compared to the normal conditions of developing. At the concentration 0.4 mg Pb% ml lead nitrate, the transplanted mulberry plants present visible phenotypic changes, with unfavorable prognosis.

The lead contaminants from the land that can be cultivated with mulberry hybrids do not have negative effect on the replanting and developing processes of the mulberry plants, these being tolerant in the interval 0.0-0.04 mg Pb% ml lead nitrate.

In these conditions it can be considered as a viable technology the phytoremediation of lead contaminated soils by utilizing the mulberry plants with sericulture destination, non-food activity. Over the concentration of 0.4 mg Pb% ml lead nitrate the plants are no longer viable.

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