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# EFFECT OF AUXIN ON PROTEIN CONTENT DURING *IN VITRO* RHIZOGENESIS OF *SYRINGA VULGARIS* L.

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**Abstract:** Protein accumulation was studied during the *in vitro* rooting of axillary shoots of *Syringa vulgaris* L. on half-strength MS medium supplemented with 7.5 mg $l^{-1}$ IBA (Indole-3-butyric acid) and on auxin free one (control). The aim of the study was to determine the most appropriate duration of auxin application by monitoring protein content as a suitable marker for the rooting of S*yringa vulgaris in vitro*. The levels of this biochemical parameter were monitored on 12$^{th}$, 24$^{th}$, 36$^{th}$, 48$^{th}$, 60$^{th}$ and the 72$^{nd}$ hour after the beginning of the experiment. Our results showed that the protein content in auxin-treated explants was generally higher than in the control, except on the 36$^{th}$ hour. The beginning of root formation was indicated by a sharp decrease in total protein accumulation on IBA containing medium. On the basis of the results the most appropriate time for a transfer of *in vitro* cultivated shoots from inductive onto expressive rooting medium is around the 36$^{th}$ hour after the beginning of the cultivation.

## INTRODUCTION

Root formation is a critical stage during the *in vitro* propagation of a number of woody species (Tzvetkova et al., 1995; Iliev et al., 2001; De Klerk, 2002; Dancheva, 2012). It was demonstrated that factors affecting the adventitious root formation are age of the donor plant (Bonga, 1982; Waldenmaier and Bünemann, 1991; Husen and Pal, 2007), the physiological state of the stock plant at the time of explant excision (Bonga, 1982; Howard, 1996; Marks and Simpson, 2000b; Marks et al., 2002), rejuvenation through repeated subculturing (Pierik, 1990; Marks and Simpson, 2000b; De Klerk, 2002; Nesterowicz et al., 2006; Economou, 2013), type and concentration of auxin (Tzvetkova et al.,

1995; Marks and Simpson, 2000a, b; De Klerk, 2002; Nesterowicz et al., 2006), macroelements and sucrose content in the rooting medium (Gaspar and Coumans, 1987; Gabryszewska, 2011; Dancheva, 2012; Economou 2013).

It was found that auxin pulse treatment could be critical for the adventitious root formation *in vitro* and the success depends on the duration of the inductive phase (De Klerk, 1996; 2001; 2002; Marks and Simpson, 2000a, b; Mitras et al., 2009; Dancheva, 2012; Lyubomirova and Iliev, 2013). However, the optimal duration of the inductive phase necessary for induction of roots also depends on the genotype of the plant (Bonga and von Aderkas, 1992; Iliev et al., 2001; De Klerk, 2002). It was also established that low concentrations of IBA (< 30 μmol) are ineffective for induction of adventitious roots in *Syringa vulgaris* L. and its cultivars and duration of 24-72 hours on medium containing 30-100 μmol IBA could induce 75-100% rooting (Marks and Simpson, 2000a; Lyubomirova and Iliev, 2013).

In most cases establishing the optimal time for transfer from inductive onto expressive medium requires continuous experiments. However, physiological parameters are reliable markers in cases when plant growth regulators (PGRs) are added in the inductive medium (De Klerk, 1996). The changes in protein content could be used as a suitable marker of the phases of rhizogenesis and their minimum values could be used for determining the optimum time for transfer (Iliev and Tzvetkova, 1995; Tzvetkova et al., 1995). A sharp decline of protein concentration at *Betula pendula* Roth. was found to precede the initiation of rooting process in the explants and could be due to the root development. It has also been reported that minimum of protein content is observed after 4-6 days depending on the type and concentration of auxin (Iliev and Tzvetkova, 1995).

The aim of this study is to establish the suitable time for transfer from inductive onto expressive medium through investigation of protein concentrations in the explants during *in vitro* rhizogenesis of *Syringa vulgaris* L.

## MATERIALS AND METHODS

*Plant material*

Axillary shoot tips obtained *in vitro* (Lyubomirova and Iliev, 2013) of approximate length of 30 mm were cultured on half-strength MS (Murashige and Skoog 1962) medium, containing 7.5 mg $l^{-1}$ IBA and on auxin free one (control). Durations of inductive phase 24, 48 and 72 hours were investigated for the rooting experiment. The number of rooted explants and total length (mm) of roots per rooted explant were determined after 5 weeks.

The protein content in the axillary shoot tips (stem and leaves) was determined after 12, 24, 36, 48, 60 and 72 hours. For both treatments the pH was adjusted to 5.5 before autoclaving at 118 kPa and temperature 120ºC for 20 min. The cultures

were grown in cultivation chamber at 23 ± 1ºC with 14 h cool white fluorescent light at photon flux density of 40 μmol $m^{-2}.s^{-1}$, daily.

*Protein assay*

Average samples (0.1 g fresh weight) were taken on the 12th, 24th, 36th, 48th, 60th and the 72nd hour after the beginning of the experiment. The plant material was homogenized in 3 ml 0.1 M phosphate buffer ($KH_2PO_4/Na_2HPO_4$) pH 7.0 and stayed in cold storage at 4°C for 30 min. After that homogenates were centrifuged of 13 000xg for 30 min. The supernatants were used as protein extracts. Protein content in the samples was measured with Perkin-Elmer Lambda 5 UV/VIS Spectrophotometer at 595 nm, using the method of Bradford (1976) and bovine serum albumin as standard.

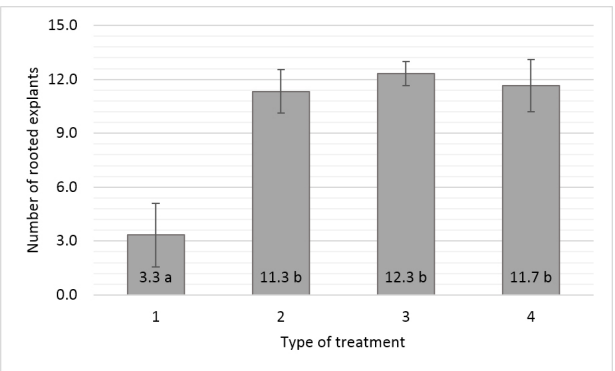
*Data analysis*

Each treatment and variant of duration of cultivation consisted of three replications, each containing 15 explants for the rooting experiment and three jars with 3 axillary shoots for the protein assay. The protein assay experiment was repeated twice.

The results were analyzed by One-Way ANOVA followed by a post hoc LSD test at $p < 0.05$, using SPSS 20.0 for Windows.

## RESULTS AND DISCUSSION

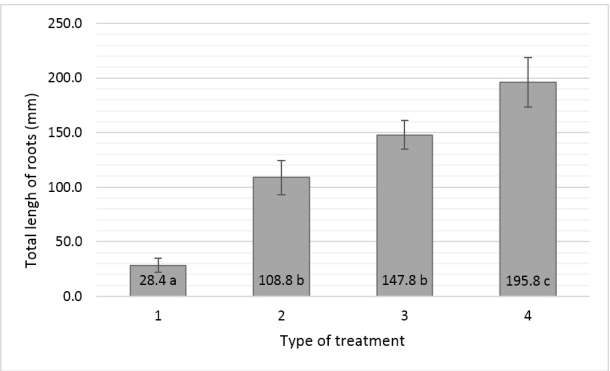
Previous experiments demonstrated that the optimum concentration of IBA for the inductive rooting phase of *Syringa vulgaris* L. shoots was 7.5 mg $l^{-1}$. Our results show that all pulse treatments (24, 48 and 72 hours) increased significantly the number of the rooted explants (between 11.3 ± 1.2 and 12.3 ± 0.7) in comparison with the control (3.3 ± 1.8). The highest number of rooted plants (12.3 ± 0.7) was observed after a 48-hour pulse treatment. However, no statistical difference was established between the three investigated durations of inductive phase (Fig 1).




Legend:
1 – Control treatment (PGRs free medium),
2 – 24-hour pulse treatment,
3 – 48-hour pulse treatment,
4 – 72-hour pulse treatment.
Vertical bars represent standard error (SE).

**Figure 1.** Average number of rooted explants.

It was shown that longer durations of inductive phase increase the number of inducted roots and their average length. Best results were obtained after 48- and 72-hour treatments. The number of roots was 2.8 ± 0.2 and 3.9 ± 0.5, and their length reached 48.4 ± 2.0 mm and 45.4 ± 1.4 mm, respectively (Lyubomirova and Iliev, 2013). However, after the longest IBA-treatment the explants formed roots with highest total length – 195.8 ± 25.8 mm. This value was statistically higher than in the other pulse treatments (108.8 ± 15.7 and 147.8 ± 13.3) and the control (28.4 ± 6.3) (Fig. 2, 3).




Legend:
1 – Control treatment (PGRs free medium),
2 – 24-hour pulse treatment,
3 – 48-hour pulse treatment,
4 – 72-hour pulse treatment.
Vertical bars represent standard error (SE).

**Figure 2.** Total length of roots per rooted explant.


**Figure 3.** Rooted explant after 35 days of cultivation on rooting medium.

The results showed that protein content remains stable with no extreme values during the 72 hours of cultivation of shoots on PGRs-free medium. Even though the protein content in the explants, cultivated on the PGRs-free medium, varied between 4.6 and 6.8 mg $g^{-1}$ FW, there was no statistical difference between these results (Table 1).

**Table 1.** Protein content during *in vitro* rhizogenesis of *Syringa vulgaris* L. (mg $g^{-1}$ FW).

| Variant | 12th hour | 24th hour | 36th hour | 48th hour | 60th hour | 72nd hour |
|---|---|---|---|---|---|---|
| | M±SE | M±SE | M±SE | M±SE | M±SE | M±SE |
| PGRs-free (control) | 6.4±0.8aA | 4.7±0.9aA | 6.8±0.8aB | 5.2±0.8aA | 4.6±0.8aA | 4.8±0.6aA |
| 7.5 mg $l^{-1}$ IBA | 7.1±0.7bcA | 6.2±2.7abA | 4.4±0.3aA | 6.8±0.8abcA | 8.4±0.9bcB | 9.3±1.4cB |

**Note:** The means (M) ± standard error (SE) within a row followed by the same small letter and in the columns followed by the same capital letter are not significantly different estimated by One-Way ANOVA followed by a post hoc LSD test at $p \leq 0.05$.

The protein content in the shoots, cultivated on the medium, supplemented with 7.5 mg $l^{-1}$ IBA, showed a steady decline during the first 36 hours of the experiment, when it reached a minimum (4.4 mg $g^{-1}$ FW). Although this value was not statistically different from the values on the 24th and 48th hour, it was the lowest of all values obtained. After the 36th hour protein content increased and at the end of the experiment reached 9.3 mg $g^{-1}$ FW. Protein content in auxin-treated explants was generally higher than in the control, except on the 36th hour, when it was under the control in the same stage of cultivation (Table 1, Figure 1).

The results obtained are a good confirmation of the biochemical studies that have been carried out so far on the metabolic changes in the rhizogenesis process (Iliev and Tzvetkova, 1995; Tzvetkova et al., 1995; Husen and Pal, 2007). These reveal that the change in protein accumulation is expressed in a decrease and then increase of protein content. The period of accumulation of protein could indicate the induction and development of root primordial (Tzvetkova et al., 1995). Our previous results show that the emergence of roots in *Syringa vulgaris* L. shoots was observed 7 days after the beginning of the experiment and by the 14th day from 40.0 to 55.6% of explants rooted (Lyubomirova and Iliev, 2013). The period of protein accumulation that starts around the 36th hour could indicate the beginning of root primordial induction that become visible after 132 more hours.

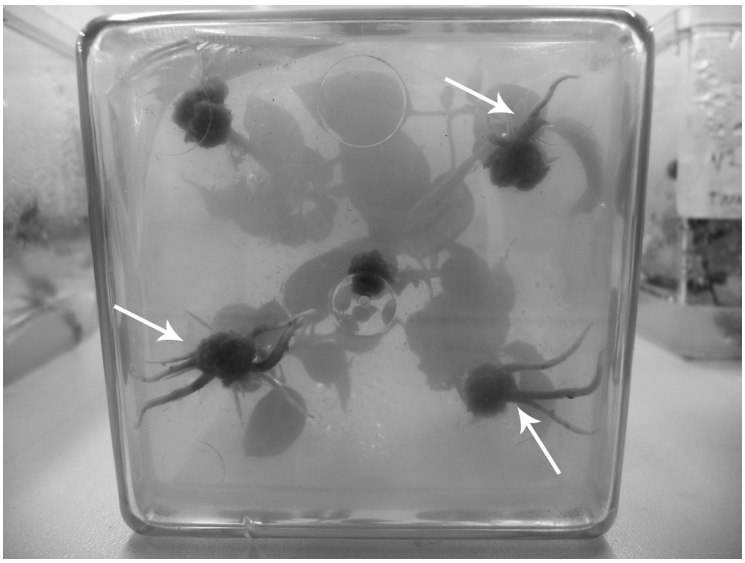


**Figure 4.** Rhizogenesis in Syringa vulgaris L. shoots 14 days after auxin pulse treatment.

Our results indicate that the optimal time for transfer of the shoots from inductive onto expressive medium is around the 36$^{th}$ hour. This is in agreement with results from the rooting experiment showing that highest number of explants rooted after 48-hour pulse treatment (Fig. 1, 2). However, the studies of Marks and Simpson (2000a) and Lyubomirova and Iliev (2013) on *in vitro* rooting of *Syringa vulgaris* L. show that incubation for 72 and 168 hours on IBA containing medium do not inhibit root formation and the outgrowth of root primordial as suggested by De Klerk et al. (1990). It was also found that the longer duration of inductive phase induces a higher number of roots (Marks and Simpson, 2000a; Lyubomirova and Iliev, 2013). The results obtained in this study show that there is a positive correlation between the duration of inductive phase and total length of roots per rooted explant. Longer exposure to IBA has also been demonstrated to result in higher protein content in the shoots that at the time of transfer could facilitate development and growth of more root primordial. This could be an effect of the particular genotype of the investigated species.

According to Husen and Pal (2007) the dynamics of the protein content in the particular variants are probably a result of interaction between the exogenous PGRs and nitrogen compounds in the explant tissues. Some of the synthetic PGRs, used for controlling the organogenesis *in vitro* are considered to form conjugates with amino-acids, thus regulating the levels of the physiologically active compounds and enzyme activities in the tissues, and to be able to effectively control the synthesis of total protein, as well as morphogenesis (Hangarter et al., 1980). It has been found that the sharp decline of protein content on the IBA containing medium during the first stages of cultivation is a result of hydrolysis

of proteins (Iliev and Tzvetkova, 1995). As a result of hydrolysis, accumulated amino-acids, amides and their secondary products, participate in the regulation of rooting process (Durzan, 1982; Iliev and Tzvetkova, 1995).

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