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EFFECTS OF *IN VITRO* PLANT AGES ON THE SUBSEQUENT GROWTH OF *Plumbago indica* L. AFTER *EX VITRO* TRANSPLANTATION

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ABSTRACT

The Indian leadwort (*Plumbago indica* L.) of the family Plumbaginaceae is a plant with high pharmaceutical value, as it contains plumbagin, a naphthoquinone with antibacterial, antifungal and anticancer properties. Among the propagation methods for the Indian leadwort, *in vitro* propagation is considered an effective method in producing disease-free transplants in a short period of time with high propagation rate. When plants grown *in vitro* are transferred to *ex vitro* condition, the environmental factors in the nursery house such as light, temperature, humidity and microorganism in the soil will affect their growth. Characteristics of transplants themselves is also critical for the subsequent growth. It is, thus, essential to establish the standards to evaluate and qualify *in vitro* plants for transplanting to *ex vitro* condition. Among these standards, the culture age of *in vitro* plants affects the maturations of their root, stem and leaves, which can in turn influence the acclimating ability and growth of *in vitro* plants after transplantation. The purpose of this study is to investigate the effects of the culture age of *in vitro* Indian leadwort plants on their performance during *ex vitro* stage. For this purpose, three different culture ages of uniform *in vitro* plants, 35, 42 and 49 day-old, were studied. After 28 days of cultivation in the nursery house under the light intensity of 70 ± 10 µmol m^{-2} s^{-1}, temperature of 35 ± 4 ^oC and relative humidity (RH) of 60 ± 10 %, all three treatments achieved 100% survival rate. Increased fresh and dry weights and percentage of dry matter after cultivation in *ex vitro* condition were not statistically different between 42 day-old and 49 day-old *in vitro* plants, but were significantly different between these plants and 35 day-old *in vitro* plants. The development of shoot and root in *ex vitro* stage of 42 day-old and 49 day-old *in vitro* plants was more balanced, as shown by the higher ratio of shoot/root dry weight, than 35 day-old *in vitro* plants. The results of this study showed that for this *Plumbago* species, bigger *in vitro* plants led to better growth during *ex vitro* stage. These results also indicated that it was possible to transfer *in vitro Plumbago* plants to *ex vitro* condition after 5 weeks of *in vitro* culture stage.

Keywords: *Plumbago indica*, *ex vitro*, *in vitro*, plant propogation, survival rate.

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INTRODUCTION

Indian leadwort (*Plumbago indica* L.), known as "Xích hoa xà" in Vietnamese, a plant of the family Plumbaginaceae, originates from India and South East Asian countries (Do et al., 2004). It is known that this species has the highest content of plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) (Mallavadhani et al., 2002). Plumbagin is found mainly in the root, but is also produced in the stem and leaves of the Indian leadwort (Galal et al., 2013; Mallavadhani et al., 2002). Many studies showed that plumbagin has cell proliferation inhibition (Panichayupakaranant et al., 2002), anticancer (Jamal et al., 2014), antifungal and antibacterial properties (Sumathy et al., 2011). Indian leadwort plant can be propagated by tubers (tuberous roots), or cuttings with the assistance of plant growth regulators (Jose et al., 2014). However, these methods require long propagation time with low multiplication rate (Pant et al., 2012). *In vitro* propagation has been used as a more efficient method than the conventional propagation to produce a large number of disease-free, uniform and genetically stable plants within a short period of time without dependence on weather condition and season (Chandra et al., 2010). A few studies on *in vitro* propagation of Indian leadwort through direct organogenesis or callus induction and regeneration using stem, leaf or root segments have been conducted (Bhadra et al., 2009; Priyanjani et al., 2019). However, when transferred to *ex vitro* condition, the survival rate of the *in vitro* plants was very low, only about 25%, mostly due to the difference of the environmental conditions of the nursery house (low humidity and higher temperature) compared to *in vitro* conditions, and the presence of microorganisms in the root environment during the initial acclimation period (Priyanjani et al., 2019). *In vitro* plants, when transferred to *ex vitro* condition (greenhouse or plastic covered nursery house), have to cope with considerable changes in environmental factors, including temperature, humidity, light intensity, etc., as well as biological factors, including the activity of the photosystem and the presence of microorganisms in the soil (Deb & Imchen, 2010). The sudden changes in living conditions can increase transpiration and weaken the plants, leading to low survival rate and slow growth in the nursery house, affecting plant production cost and the application of micropropagation in transplant production (Pospíšilová et al., 2007). Many studies tried to increase *in vitro* plant aclimation ability during transplanting to *ex vitro* condition by controlling *ex vitro* environmental factors, improving *in vitro* culture condition, etc. Some studies aimed at controlling the opening and closing of stomata, investigating the changes in chloroplast structure and metabolism of *in vitro* plants after transplanting to *ex vitro* condition (Bolar et al., 1998; Pospíšilová et al., 2007). However, few studies have investigated the suitable age of *in vitro* plants at which they can withstand the changes in living environment when transferred to *ex vitro* condition. The age of *in vitro* plants indicates the maturation in function of shoot and root, and also affects the growth and development of plants under partially or completely controlled environment in the nursery. Thus, it is essential to identify the suitable age of *in vitro* Indian leadwort plants for transplanting to *ex vitro* condition. Not only does it provide more insights into adaptation of growth and development of Indian leadwort plants during transition from *in vitro* to *ex vitro* condition, it also helps to establish an important step in the procedure for micropropagation of Indian leadwort, offering significant economic benefits when applied to practical production.

MATERIALS AND METHODS

Plant materials used in this experiment were *in vitro* Indian leadwort plants, originating from Yok Don National Park (Dak Lak Province, Vietnam). The *in vitro* plants were cultured on MS macro-and micro-nutrient solution (Murashige and Skoog, 1962), supplemented with 10 g L^{-1} sucrose (Bien Hoa Sugar Co., Dong Nai Province, Vietnam),

7 g L^{-1} agar (Ha Long Food Co., Hai Phong Province, Vietnam) and Morel & Wetmore vitamin solution (Morel & Wetmore, 1951), in plastic bag-type vessels (V = 1 L when inflated with air) having two ventilated paper filter discs (Nguyen et al., 2016). Each vessel contained 6 shoots having two open leaves with 100 mL culture solution. The plants were cultured under photosynthetic photon flux density (PPFD) of 35 ± 5 μmol m^{-2} s^{-1}, provided by white fluorescent lamps (Dien Quang Co., Ho Chi Minh City, Vietnam), with photoperiod of 12 h/day, air temperature of 25 ± 2 ºC, relative humidity (RH) of 55 ± 5%. PPFD was measured with a LI-250A light meter coupled with a LI-190R quantum sensor (LI-COR® Inc., Lincoln, USA).

Ten each of three different *in vitro* culture ages of Indian leadwort plants, 35, 42 or 49 day-old at the time of transplant and having an average of 4, 5 and 6 leaves, respectively, were taken out from culture bags on the same day, and the experiment was replicated three times. Average initial fresh weights of 35, 42 and 49 day-old plants were 320, 800 and 950 mg per plant, respectively, and the average initial dry weights were 35.5, 70.1 and 85.6 mg, respectively. The roots were washed clean under running tap-water to remove agar, then the plants were quickly transplanted into plastic pots (ø = 8 cm, height = 13 cm) containing Klasmann growing substrate (Klasmann-Deilmann GmbH, Geeste, Germany) mixed with perlite (Cell Green Co., Binh Duong province, Vietnam) at the ratio of 5:1, and put in nursery house with daily average PPFD of 70 ± 10 μmol m^{-2} s^{-1}, average temperature of 35 ± 4 ºC, average RH of 60 ± 10%. During the first four days after transplanting, the humidity around the plants were maintained by a Faran humidifier (Faran Industrial Co., Ltd., Gyeonggi-do, South Korea). From the fifth day onward, the plants were watered three times/day (total 200 mL/day per plant) using a drip irrigation system (Netafim, Tel-Aviv, Israel). From the eighth day onward, the plants were fertilized with dissolved NovAcid fertilizer (19-19-19, EC = 1.0 dS m^{-1}, pH = 5.5–6.5) (Israel Chemicals Ltd., Tel-Aviv, Israel), applied through the drip irrigation 3 times/week.

On days 14 and 28 after transplantation, the growth parameters of the plants were measured, including survival rate, fresh (FW) and dry weights (DW), number of leaves (NoL), shoot length, root length and leaf area (LA) (measured using a LI-3100C area meter, LICOR® Inc., Lincoln, USA). The bioassays for ascertaining relative activity of endogenous hormones (auxin and cytokinin) in Indian leadwort plants were carried out on day 28 according to the method developed by Nguyen et al. (2003). Increased fresh and dry weights (IFW, IDW) of plants was calculated by deducting average initial fresh and dry weights from the fresh and dry weights measured on days 14 and 28. Percentage of dry matter (% DM) was a proportion of dry weight to fresh weight measured on days 14 and 28. Relative growth rate (RGR) was calculated based on the equation of Hoffmann & Poorter (2002):

$$RGR = \frac{\overline{\ln W_2} - \overline{\ln W_1}}{t_2 - t_1}$$

In which W_1 and W_2 were dry weights of Indian leadwort plants at time t_1 and t_2 (day), with $t_2 > t_1$.

One-way ANOVA test was performed on the data using STATGRAPHICS Plus 3.0 software for Windows. For parameters with significant differences among treatments, means of three treatments were ranked by least significant difference (LSD) test at $p \leq 0.05$, 0.01 or 0.001. Graphs were made with Microsoft Excel 2019.

RESULTS AND DISCUSSION

In vitro Indian leadwort plants had, regardless of the culture ages, 100% survival rate 14 and 28 days after transplantation to *ex vitro* condition, and increased in biomass while culturing in the nursery house (Fig. 1, Table 1).

The difference in age of *in vitro* plants was reflected to the differences of the weight and

NoL of the plants at the time of transplantation. These differences could influence the ability to absorb sun light by the leaves for photosynthesis and nutrients in the soil by the roots for the metabolic processes necessary for the growth of the plants. Besides, the difference in NoL affects the total amounts of light and CO_2 captured for photosynthesis (Donnelly et al., 1985). D35 old plants had the lowest IFW, IDW, NoL and LA compared to the other two ages (Table 1, Fig. 2).

According to Weraduwage et al. (2015), the relationship between growth and leaf development is not linear. It depends on the distribution of carbohydrates between leaves and roots. Root development is related to water and nutrient absorption, and, as a result, affects the photosynthetic rate of plants, especially the activity of photosystem II (Zakaria et al., 2020). Therefore, IFW, IDW and NoL of D42 and D49 plants were not significantly different on both day 14 and day 28 after transplantation. LA of D49 plants was wider (46.5 cm^2) than that of D35 and D42 plants on day 14 day after transplantation. However, 28 days after transplantation, LA (71.8 cm^2) of D49 plants was lower than that (74.1 cm^2) of D42 plants (Table 1).

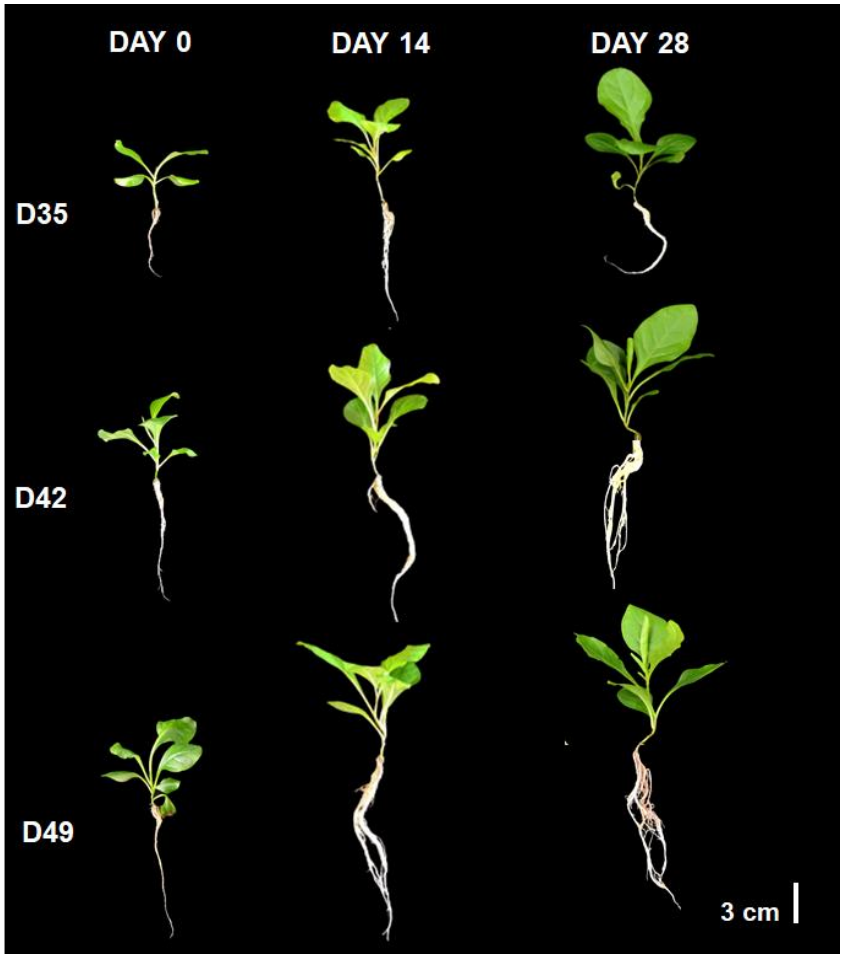

Figure 1. Growth of *in vitro Plumbago indica* L. plants of different ages 14 and 28 days after transplantation into *ex vitro* stage in the nursery house.

Notes: For the age codes, D35, D42 and D49 refer to the ages (days) of *in vitro* plants at the time of transfer to the *ex vitro* stage.

Table 1. The increases of fresh weight (IFW) and dry weight (IDW), number of leaves (NoL) and leaf area (LA) of *in vitro Plumbago* plants 14 (D14) and 28 (D28) days after transplantation to *ex vitro* condition

Age[z]	IFW (mg/plant)		IDW (mg/plant)		NoL (leaves/plant)		LA (cm^2)	
	D14[y]	D28	D14	D28	D14	D28	D14	D28
D35	259.3 b[w]	795.6 b	55.1 b	105.7 b	5.4 b	6.3 b	21.9 c	33.6 c
D42	546.2 a	1601.9 a	142.9 a	245.0 a	6.2 a	7.9 ab	41.2 b	74.1 a
D49	516.8 a	1548.8 a	152.1 a	252.5 a	6.6 a	8.8 a	46.5 a	71.8 b
ANOVA[x]	***	***	***	***	*	**	***	***
CV (%)	2.5	1.3	4.5	1.3	6.0	6.3	2.8	0.6

Notes: [z]: For the age codes, see Fig. 1; [y]: Abbreviations of D14 and D28 refer to measuring days; [x]: *, **, or ***: Significant at $p \leq 0.05$, 0.01 or 0.001 respectively; [w]: Means in the same column followed by the same letters are not significantly different according to LSD-test.

Figure 2. Leaf canopy of *in vitro Plumbago* plants 14 (upper) and 28 (lower) days after transplantation to the *ex vitro* condition in the nursery house
Notes: For the age codes, see Fig. 1.

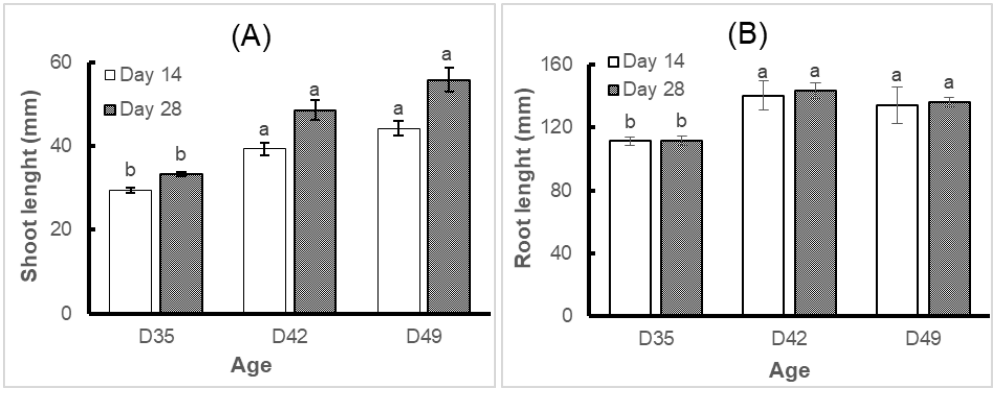

Figure 3. (A) Shoot length and (B) Root length of *in vitro Plumbago* plants 14 and 28 days after transplantation to the *ex vitro* condition in the nursery house
Notes: For the age codes, see Fig. 1; Error bars: SD.

Similar to the weight increases in table 1, on both day 14 and 28 after transplantation, shoot length and root length of Indian leadwort plants of ages D42 and D49 of in vitro culture were not significantly different from each other, but were both statistically higher than those of age D35 (Fig. 3). Shoot lengths of all three ages of *in vitro* plants increased over cultivation time in the nursery house (Fig. 3A). However, root lengths of all ages did not increase from day 14 to day 28 of *ex vitro* cultivation (Fig. 3B).

On both 14 and 28 days after *ex vitro* transplantation, shoot/root fresh weight ratios of D35 plants were significantly higher (3.2 and 6.9, respectively) than those of D42 (2.6 and 4.5, respectively) and D49 (2.5 and 4.4, respectively) plants (Fig. 4). Similarly, shoot/root dry weight ratios of D35 were significantly higher (4.7 and 7.3, respectively) than those of D42 (3.5 and 4.9, respectively) and D49 (3.7 and 5.1, respectively) plants (Fig. 4). This indicated imbalance between shoot and root growth of D35 plants. This imbalance became larger over cultivation time in the nursery house, with shoot/root fresh and dry weight ratios of D35 plants increased by 2.1 and 1.5 times, respectively, by 28 days of *ex vitro* cultivation compared to that of 14 days of cultivation. We suspected that this might be due to the effect of endogenous auxin and cytokinin produced by plants during their growth in the *ex vitro* stage. In fact, the auxin/cytokinin ratios of D35, D42 and D49 *Plumbago* plants after 28 days in *ex vitro* cultivation were 0.64, 1.32 and 1.30, respectively, based on the bioassay measurements (Table 2). Higher auxin/cytokinin ratio will promote root formation, resulting in more balanced shoot and root growth.

Table 2. Relative activity of endogenous auxin (IAA) and cytokinin (zeatin) of *in vitro* *Plumbago* plants in the *ex vitro* cultivation measured by bioassays on day 28

Age[z]	Relative activity (mg/L)		
	Zeatin	IAA	Auxin/Cytokinin
D35	0.19 a[x]	0.13	0.64 b
D42	0.12 b	0.16	1.32 a
D49	0.11 b	0.14	1.30 a
ANOVA[y]	***	NS	**
CV (%)	7.7	12.9	15.3

Notes: [z]: For the age codes, see Fig. 1; [y]: NS, *, **, or ***: Non-significant or significant at $p \leq 0.05$, 0.01, or 0.001 respectively; [x]: Means in the same column followed by the same letters are not significantly different according to LSD-test.

Shoot/root weight ratio varies depending on plant age, water, nutrients and light availability, etc. The change in the environmental factors such as nutrients, light and CO_2 can alter growth pattern of plants (Ericsson, 1995; Bonifas & Lindquist, 2006). Plants respond to changes of environmental conditions by regulating the balance in nitrogen and carbon partitioning (Chen et al., 1993). The ratio between shoot and root weights can be altered corresponding to the carbon and nitrogen partitioning levels in root and shoot (Marcelis, 1996). Both fresh and dry shoot/root weight ratios of D42 and D49 plants were lower than those of D35 plants at 14 and 28 days after *ex vitro* transplantation (Fig. 4). This showed that the developments of roots of D42 and D49 were more robust than that of D35 plants. Indian leadwort plants of D42 and D49 *in vitro* plants had higher initial fresh weight as well as more leaves than did D35 plants, which translated into better carbon fixation and root formation after *ex vitro* cultivation. Consequently, these plants would adjust the absorption of nutrients from root to ensure a balanced growth, since

photosynthetic rate is directly related to nitrogen content in leaves (Agren & Ingestad, 1987; Hilbert, 1990; Levin et al., 1989; Lindquist, 2001).

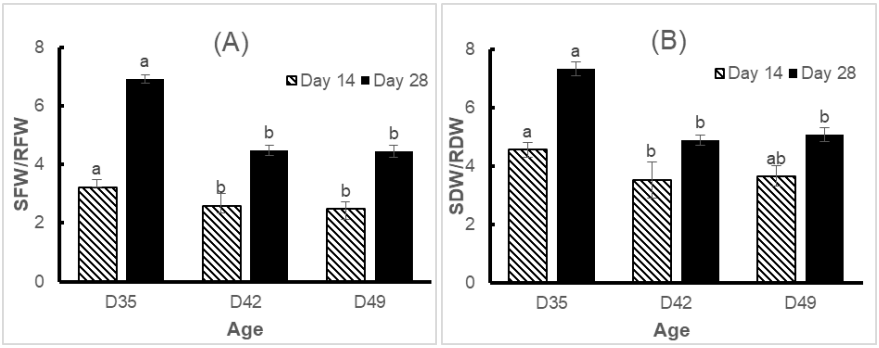

Figure 4. (A) Shoot fresh weight/Root fresh weight (SFW/RFW) and (B) Shoot dry weight/Root dry weight (SDW/RDW) of *in vitro Plumbago* plants in the *ex vitro* stage 14 and 28 days after transplantation in the nursery house.
Notes: For the age codes, see Fig. 1; Error bars: SD.

On day 28 after *ex vitro* transplantation, % DM of Indian leadwort plants followed a similar trend as plant DW change, lowest in D35 plants (12.3%) while comparable between in D42 and D49 plants (13 and 13.3%, respectively) (Fig. 5). Plant growth is typically characterized by relative growth rate (RGR); however, RGR has a tendency to decline as plants become larger, and this makes it difficult to compare plant species of different sizes. Relative growth rate of Indian leadwort plants during 28 days of cultivation in the nursery house was not different statistically between D35 plants (0.051 d^{-1}) and D42 plants (0.053 d^{-1}), while that of D49 (0.048 d^{-1}) was significantly lower than others (Fig. 5). According to Li et al. (2014), RGR is proportional to shoot weight, but inversely proportional to root weight, and also depending on plant age, water and nutrient availability and light intensity. In this series of experiments, Indian leadwort plants of D35 had shoot/root weight ratios higher than those of D49 plants (Fig. 4). Meanwhile, on day 28 after transplantation, even though shoot/root weight ratios of D42 plants were not different from those of D49 plants (Fig. 4), LA of D42 plants was greater than that of D49. This showed that thanks to higher RGRs, D35 and D42 plants distributed more carbohydrates into leaves for photosynthetic activity.

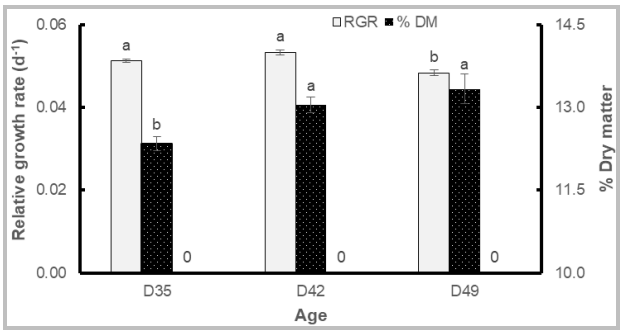

Figure 5. Relative growth rate (RGR, d^{-1}), and percentage of dry matter (% DM) of *in vitro Plumbago* plants 28 days after *ex vitro* cultivation.
Notes: For the age codes, see Fig. 1; Error bars: SD.

In summary, *in vitro* Indian leadwort plants at all ages (35, 42 and 49 day-old) could survive 100% after *ex vitro* transplantation without any acclimatization step. *In vitro* plants were able to adapt and develop when transferred to *ex vitro* condition at the *in vitro* culture age of 35 day-old. Older *in vitro* plants had faster growth when transplanted to *ex vitro* condition, but there was no significant difference in growth between *in vitro* plants of 42 and 49 days old. During the observation of 28 days in the nursery house, 42 day-old plants showed the best growth after transplanted to *ex vitro* condition.

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