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Cuttings of *Lippia alba* with emphasis on time for seedling formation, substrates and plant growth regulators

Cristina B Lima; Ana C Boaventura; Marli M Gomes

UENP-CLM, CCA, C. Postal 261, 86360-000 Bandeirantes-PR; crislima@uenp.edu.br; ac_boaventura@hotmail.com; marlimoraes06@hotmail.com

ABSTRACT

We aimed to establish the period of time required for seedlings formation, as well as to evaluate the effect of substrates and plant growth regulators in cuttings of *L. alba*. Three experiments were performed, with statistical designs chosen according to each test objectives. Rooting (%), shoot height (cm), longest root length (cm), leaves number, fresh and dry matter masses (g), were analyzed. The absolute rate of shoot and root system growth, together with the relationship between dry mass of roots and shoots were efficient in predicting the period of greatest speed and accumulation of organic matter in plants. The plant growth regulators favored the vegetative performance of seedlings, with better development 30 days after the cuttings containing mineral fertilizers, vermicompost, clay soil and sand commercially known as 'medium sand'.

Keywords: *Lippia alba*, propagation, IBA, rooting.

RESUMO

Estaquia de erva cidreira com ênfase no tempo para formação das mudas, substratos e enraizadores

O presente trabalho teve por finalidade estabelecer o período de tempo necessário para a formação de mudas, bem como, avaliar o efeito de substratos e produtos enraizadores na estaquia de *Lippia alba*. Para tanto, foram realizados três experimentos individuais, com delineamentos estatísticos escolhidos, conforme os objetivos de cada ensaio. Foram analisados o enraizamento (%), altura da parte aérea (cm), comprimento da maior raiz (cm), número de folhas, massas das matérias frescas e secas (g). A taxa de crescimento absoluto da parte aérea e sistema radicular, aliada à relação entre massa da matéria seca de raízes e da parte aérea foram eficientes em predizer o período de maior velocidade e acúmulo de matéria orgânica nas plantas. O emprego de produtos enraizadores favoreceu o desempenho vegetativo das mudas, que atingiram melhor desenvolvimento trinta dias após a estaquia, em substrato contendo fertilizantes minerais, vermicomposto, solo argiloso e areia comercialmente conhecida como 'areia média'.

Palavras-chave: *Lippia alba*, propagação, AIB, enraizamento.

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The species *Lippia alba*, popularly known as lemon balm, is a promising source of raw material for cosmetic, pharmaceutical and flavoring industries (Ehlert, 2003). The antiprotozoal, bactericidal and antifungal activities of this plant (Tavares *et al*., 2011) make it possible to be used in the preparation of pesticides to control plant pathogenic diseases. Features, like rusticity and wide phenotypic plasticity (Yamamoto, 2006), reinforce its industrial potential, however available agronomic information in scientific literature are not enough to support the commercial cultivation of this plant.

Vegetative propagation method, when used for medicinal plants, prevents abrupt changes in active principle contents, keeping the final product quality (Pedrosa *et al*., 2010). Cutting multiplies and preserves varietal characteristics of mother plants quickly and with greater economic viability. However, technical aspects of rooting cuttings as substrates, containers, plant growth regulators and time of permanence of seedlings at nursery should be evaluated for each species. Interaction between endogenous factors and environmental conditions (Fachinello *et al*., 2005) should be also considered.

The ideal substrate must be accessible, with attention directed to physical properties, which cannot be corrected during seedling formation and, according to Barjón *et al*. (2004), must contain particle size distribution sufficient to maintain high total porosity, easily available water, aeration, low density and stable structure which prevent the contraction or expansion of the substrate in the container, allowing proper stake support.

For Pimenta *et al*. (2007), the growth of plant organs, especially the roots, is among the biological functions of auxins, helping seedling establishment, mainly, in less favorable times for the plant development in the field. The indole butyric acid (IBA) is considered the most effective synthetic auxin to promote rooting, due to its low toxicity, stability to action of light, and great resistance to attack by biological action (Biasi & Costa, 2003).

The ideal age for planting seedlings is determined by empirical values, due to lack of appropriate procedure for this purpose. The planting of "overaged" seedlings leads to failures in establishment and reduction in the initial growth in the field, because if the seedlings stay for a long time in the nursery, vegetative growth reduces and tends to root system folding (Mafia *et al*., 2005). In this context, we aimed to establish the period of time necessary for seedling formation through vegetative propagation using cutting, as well as, to evaluate the effect of the substrates and rooting products on cuttings of *Lippia alba.*

MATERIAL AND METHODS

The experiments were carried out from September, 2012 to February, 2013, with stakes prepared from branches taken from mother plants, from the medicinal plant collection of the Universidade Estadual do Norte do Paraná (UENP/CLM), in the municipality of Bandeirantes-PR (23º17'S, 50º09'W, altitude 419 m). Botanical identification of mother plants was performed by technicians of Museu Botânico de Curitiba (PR), based on the herbarium specimens, which was incorporated into the collection of Centro de Educação e Pesquisa Ambiental of UENP/CLM, after identification, under the registration number 534. After branch collection, performed in the morning, the leaves were removed and the stakes were standardized in size of 20 cm length, 0.5 cm diameter (Biasi & Costa, 2003), 4 to 6 gems and 3 to 4 grams, with a bevel cut at the bottom. Three experiments were carried out using this material; the stakes were planted in transparent polyethylene containers (300 mL capacity), kept in a greenhouse and watered daily, when necessary. The evaluations of experiments 1 and 2 were performed 20 days after cutting, and the composition described in experiment 1 was used as substrate in experiments 2 and 3.

Experiment 1 (substrate evaluation) – cutting was performed immediately after the making of the stakes, using as substrates materials of easy purchasing and availability in the region: pure sand commercially known as 'medium sand' (substrate 1) and, a composition (substrate 2) composed with the following materials and volumetric proportions: vermicompost produced at UENP/CLM Farm School (2), soil for construction (subsoil at least 60 cm depth) clayey texture: 69.8% of clay, 21.9% of sand and 8.3% of silt (4) and sand commercially known as 'medium sand' (2). Determination of soil texture was performed at the Laboratory of Soil Analysis of UENP/CLM. Classification of the predominant soil in the municipality of Bandeirantes-PR is typic eutroferric red latossol (Embrapa, 2006). For each 8 liters of this composition, 0.18 of urea, 2.77 of superphosphate and 0.55 g of potassium chloride were added. This mixture is usually used for preparing seedlings in the nursery of UENP/CLM, the addition of fertilizer being made empirically, situation repeated in small farms in the municipality, whenever soil for construction is used. Physical and particle size analyzes of substrates were performed by Substrates Analysis Laboratory for Plants at Universidade Federal do Rio Grande do Sul and substrate chemical analyses by Laboratório Santa Rita (PR). The results of these analyses can be found in Table 1.

Experiment 2 (IBA x concentrations) – two sources of auxins were used: IBA p.a, (100%) and rooting talc marketed by LR Paisagismo (3 g/L of IBA + 1 g/L of thiamine + 20% of sulphur + excipient Q.S.P.: 20 g). The auxin treatment was prepared via solution in the following IBA and rooting talc concentrations: 0, 10, 30, 60, 90 and 120 mg/L. The basal region of the stakes remained immersed in the solution for 24 hours (Hartman *et al*., 2002). At zero dose, conventional technique of cutting was adopted, and the stakes planted immediately after preparation.

Experiment 3 (period of seedling formation) – stakes were planted immediately after the preparation, with evaluations performed periodically, 20, 30, 40, 50 and 60 days after cutting. Evaluations were carried out by observations of shoot and root dry matter masses in order to calculate absolute growth rate (AGR) of the seedlings, during the evaluations. AGR (g/day) was calculated using the equation: AGR = (M2-M1)/(T2-T1), where: M= mass of dry matter; T= time.

In experiments 1 and 2 we evaluated the rooting percentage, shoot height (cm), longest root length (cm), shoot and root fresh and dry matter to calculate AGR. The length of roots was measured using a graduated ruler in millimeters. Root fresh mass was obtained by removing the roots with a stylus and weighing on a precision scale. To determine dry matter mass, the plant material was packed in paper bags, individually separated per stake and dried in a forced air circulation oven, at temperature of 60°C, until constant mass.

A completely randomized design was used, with 4 replications containing 10 stakes each, for each type of substrate and evaluation period. Experiment 2 was carried out in a factorial scheme 2 x 6 (product rooting x concentrations). Data were subjected to variance analysis and averages analyzed by Scott-Knott test ($p \leq 0.05$). The effects of rooting concentrations (experiment 2) and period of seedling formation (experiment 3) were also evaluated using regression analysis. Analyses were carried out using statistical software Sisvar® (Ferreira, 2011).

RESULTS AND DISCUSSION

Experiment 1 (substrate evaluation) - For most physical characteristics, substrates did not reach the limits referred to as ideal in scientific literature (Table 1). Substrate 2 showed higher values comparing with substrate 1 in relation to organic matter, CEC, sum and base saturation. In particle size of substrate 1 particles between 0.5 and 0.1 mm are predominant and in substrate 2 between 1.40 to 0.1 mm favoring higher percentages of current moisture,

Table 1. Chemical, physical and particle size analysis in the substrates used for *Lippia alba* cuttings (características químicas, físicas e análise granulométrica dos substratos utilizados para estaquia de *Lippia alba*). Bandeirantes, UENP/CLM, 2013.

Substrates	Chemical characteristics										
	pH	CE	MO	P	K	Ca	Mg	H+Al	SB	CTC	V
	$CaCl_2$	(mS/cm)	(g/kg)	(mg/dm³)	(cmol$_c$/dm³)						(%)
1	6.3	0.03	2.70	11.4	0.07	1.3	0.7	1.66	2.07	3.73	55.5
2	6.5	0.20	24.20	89.8	1.60	9.4	4.2	2.80	15.20	18.00	84.7

	Physical characteristics											
	DU	DS	UA	PT	EA	AFD	AT	AR	AD	CRA(10)	CRA(50)	CRA(100)
	(kg/m³)		(%)									
1	1687.08	1683.81	0.19	42.8	5.94	29.55	3.19	4.13	32.74	36.86	7.31	4.13
2	1319.20	1249.10	5.30	59.2	7.10	24.00	5.60	22.40	30.20	52.10	28.00	22.40
Ideal[1]	-------	400-500	----	>85	30	20-30	4-10	25-30	24-40	55-70	31-40	25-31

	Particle size analysis (mm)					
	>3.35	3.35-2.00	2.00-1.40	1.40-0.50	0.50-0.10	<0.10
1	0.0	0.0	0.0	9.1	89.5	1.2
2	1.0	5.0	4.3	28.5	58.6	2.3

Substrates 1= pure sand (A) (areia pura) and 2= mixture with vermicompost, soil, sand and mineral fertilizers (composição feita com vermicomposto, solo, areia e fertilizantes minerais); EC= electrical conductivity (condutividade elétrica); OM= organic matter (matéria organica); DU= wet density (densidade úmida); DS= dry density (densidade seca); UA= Current humidity (umidade atual); PT= total porosity (porosidade total); EA= aeration space (espaço de aeração); AFD= easily available water (água facilmente disponível); AT= buffering water (água tamponante); AR= remaining water (água remanescente); CRA (10), (50) and (100)= water retention capacity under suction 10, 50 and 100 cm of water column determined volume basis (capacidade de retenção de água sob sucção de 10, 50 e 100 cm de coluna de água determinado em base volumétrica); AD= water available that can be obtained by adding AFD + AT (água disponível que pode ser obtida pela soma de AFD + AT); [1]ideal values referenced as Barjón *et al*. (2004), and Schmitz *et al*. (2002) {valores ideais referenciados conforme Barjón *et al*. (2004) e Schmitz *et al*. (2002)}.

Table 2. Shoot height, length of roots, leaves numbers and fresh weight of seedlings of *Lippia alba* after cutting in different concentrations of thiamine and AIB {altura da parte aérea, comprimento da maior raiz, número de folhas e massa da matéria fresca de mudas de *Lippia alba* desenvolvidas por estaquia em diferentes concentrações de tiamina e AIB}. Bandeirantes, UENP/CLM, 2013.

Characteristics	Product	Concentrations (mg/L)					
		0	10	30	60	90	120
Height of aerial part (cm)	Talc	11.7 Ba	15.1 Aa	14.1 Aa	14.1 Aa	14.0 Aa	13.6 Aa
	AIB	11.7 Ca	16.5 Aa	12.0 Cb	13.4 Ba	14.1 Ba	9.3 Db
Length of the longest root (cm)	Talc	16.3 Aa	16.3 Aa	16.7 Aa	16.5 Aa	16.1 Aa	15.5 Aa
	AIB	16.3 Aa	14.0 Ab	12.7 Bb	14.4 Ab	15.7 Aa	10.7 Bb
Number of leaves	Talc	25.4 Ba	28.2 Ba	28.1 Ba	31.5 Aa	32.7 Aa	30.4 Aa
	AIB	25.4 Aa	24.6 Aa	26.4 Aa	25.5 Ab	27.0 Ab	21.4 Ab
Fresh matter mass (g)	Talc	6.79 Aa	6.71 Ab	6.27 Aa	7.25 Aa	6.48 Ab	6.53 Aa
	AIB	6.79 Ba	8.84 Aa	7.10 Ba	7.57 Ba	8.91 Aa	6.76 Ba
Adjustment of cubic regression							R^2
Rooting percentual		$\hat{Y} = 107.083-10.586X+3.829x^2-0.405X^3$*					87%
Length of the longest root (cm)		$\hat{Y} = 21.339-6.941X+2.289X^2-0.226X^3$**					94%

Means followed by different letters, uppercase in line and lowercase in column differ at 5% according to Scott-Knott test; significant at 5% (*) or 1% (**) by F test {médias seguidas por letras distintas, maiúsculas na linha e minúsculas na coluna, diferem entre si a 5% de acordo com Scott-Knott; significativo a 5% (*) ou 1% (**) pelo teste F}.

total porosity, aeration space, available water and water retention capacity. The percentage of organic matter in substrate 2 exceeded the percentage verified in substrate 1, however this value was lower than the minimum of 80% (Barjón *et al*., 2004), recommended for substrates used for seedling production in containers. Schmitz *et al*. (2002)

Figure 1. Seedlings of *Lippia alba* after cutting in substrates 1= pure sand (A) and 2= mixture with vermicompost, soil, sand and mineral fertilizers (B) {mudas de *Lippia alba*, após a estaquia nos substratos 1= areia pura (A) e 2= composição feita com vermicomposto, solo, areia e fertilizantes minerais (B)}. Bandeirantes, UENP/CLM, 2013.

verified that the addition of organic elements to substrates prepared with soil and sand was not enough to increase the percentage of organic material to the minimum value established as optimal.

The mixture prepared with vermicompost, soil, sand and mineral nutrients (substrate 2), provided the best medium for *L. alba* seedlings formation (Figure 1), with an average of 100% for rooting, 11.7 cm for shoot height, 16.3 cm for longest root length, 25.4 leaves, 6.825 g of fresh matter mass and 1.725 g of dry matter mass, significantly higher than those observed in sand. These results showed that physical and chemical properties of this mixture, even different from those considered optimal provided a satisfactory development for cuttings of *L. alba*.

Substrates interfered in vegetative propagation by cuttings of L. *alba*, as its constitution, and its effects reported in the work of Ehlert (2003), in which substrate based on soil, manure and carbonized rice husk differed in relation to shoot and root dry mass. Biasi & Costa (2003) reported that similarities related to rooting percentage, sprouting and number of buds per stakes in rice husk substrates, Plantmax®, vermiculite and soil. Tavares *et al*. (2012) indicated mixtures of Plantmax and carbonized rice straw and Plantmax and tanned cattle manure, for having provided the best results for leaf and stem fresh and dry matter masses.

Aeration is essential for root respiration and plant growth. According to Barjón *et al*. (2004), however, local environmental conditions, where particular plant species grow spontaneously, determine desirable characteristics of a substrate for growing it. In this sense, lemon balm plant grows on the banks of rivers, dams, lakes and ponds in tropical, sub-tropical and temperate regions (Stefanini *et al*., 2002); stakes taken from this plant may originate quality seedlings, in substrates with higher density and water availability, in relation to porosity and aeration space.

Experiment 2 (IBA x concentrations) - Interaction between IBA sources and concentrations for shoot height, largest root length, leaf number and fresh matter mass averages was noticed (Table 2). For percentage of rooting stakes, rooting products did not differ from dose zero which showed average of 100%, showing original capacity of cuttings of *L. alba* rooting. Organic matter accumulation was potentiated by concentrations of two products, except for the dose of 120 mg/L, when average of this variable decreased significantly.

Shoot growth was benefited by application of rooting promoters in concentration of 10 mg/L (Table 2). The effect of talc-based rooting on the largest root length equaled to the control and exceeded IBA (except in 90 mg/L). The talc-based rooting increased the number of seedling leaves in doses from 60 to 120 mg/L; however, this increase was not enough to exceed the average of the control in relation to fresh matter mass. In this case, IBA was responsible for the best results in doses of 10 and 90 mg/L.

Cubic regression model showed significant effects on the product concentrations for the percentage of rooting and largest root length with higher and lower average peak (Table 2). Rooting was of 100% in concentrations of 0.30 and 90 mg/L and minimum (93.75%) in 120 mg/L. Rooting products did not benefit the largest root growth, as the highest average for this variable was observed in seedlings, whose stakes did not receive application of the products (16,5 cm) and the lower (13,1 cm) occurred in 120 mg/L.

Albuquerque *et al*. (2001) verified negative effect in concentrations ranging from 250 to 2000 mg/L of IBA in aboveground and root dry matter mass of *L. alba* seedlings obtained through cutting, concluding that it is not necessary using this product in stakes of this plant. According to Lone *et al*. (2010), exogenous supply of auxin, can promote a hormonal change, which, sometimes, is not favorable for rooting of cuttings. However, Lolli (2001) concluded that formation of roots in semi-hardwood cuttings of *L. alba*, with leaves, was favored by the application of 2000 mg/L of IBA. According to Pizzatto *et al*. (2011), an optimal level of concentration of exogenous auxin to stimulate the growth and differentiation of root tissues can be verified and Fachinello *et al*. (2005) complemented that this level depends on auxin concentration in the stake. Nevertheless, this stimulation occurs up to a certain concentration, differing for each species, from which the effect becomes inhibitory.

Experiment 3 (period of seedling formation) - The authors verified 100% of rooting of cuttings, during the five periods of evaluations. The variables shoot height, largest root length, shoot and root fresh and dry matter masses were adjusted to quadratic regression model, with a tendency of increment reduction, sixty days after cutting

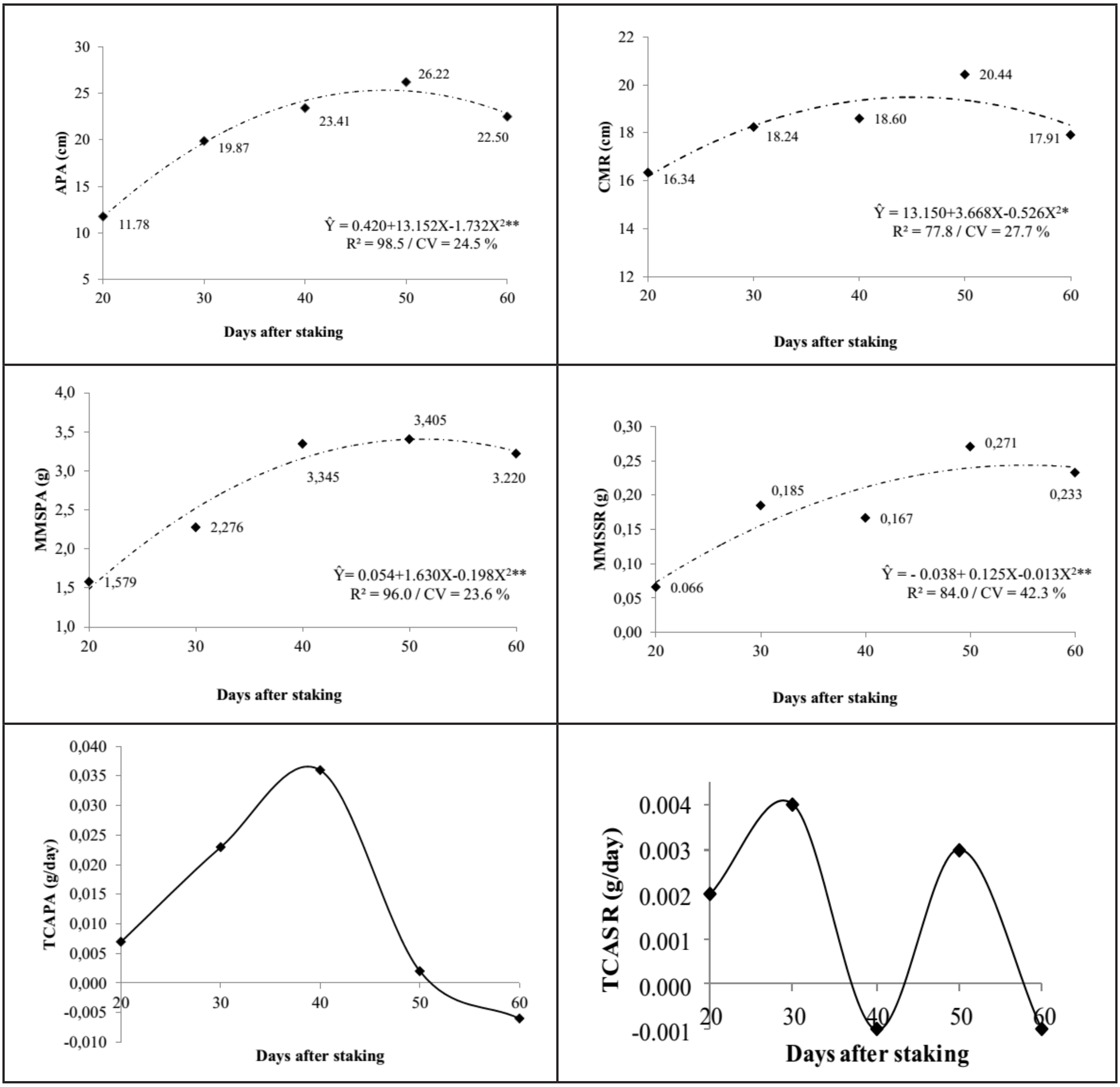

Figure 2. Shoot height (APA), length of roots (CMR), shoot dry weight (MMSPA), root dry weight (MMSSR), absolute growth of shoots (TCAPA) and absolute growth of root system (TCASR) of *Lippia alba* seedlings at 20, 30, 40, 50 and 60 days after cutting {altura da parte aérea (APA), comprimento da maior raiz (CMR), massa da matéria seca da parte aérea (MMSPA), massa da matéria seca do sistema radicular (MMSSR), taxa de crescimento absoluto da parte aérea (TCAPA) e taxa de crescimento absoluto do sistema radicular (TCASR) de mudas de *Lippia alba* aos 20, 30, 40, 50 e 60 dias desenvolvidas por estaquia}. Bandeirantes, UENP/CLM, 2013.

(Figure 2). Peak of growth occurred fifty days after the cutting, with increases of 122.6% for average of shoot height, 25.1% for largest root length, 115.6% for shoot dry matter and 310.6% for root system dry matter, in relation to the evaluation performed on the 20th day.

Shoots and roots grew simultaneously up to 30 days after cutting (Figure 2). At forty days, the increase rate of dry matter decreased in root system and increased in shoot area. This pattern is different, in the following evaluation and at sixty days is negative both for shoot and for the root system.

Increment peaks do not always correspond to the best quality of the seedling, as according to Minami (2010), an abundant increase in shoot area may indicate old seedlings or, developed in hot weather conditions. According to Alfenas *et al*. (2004), time of permanence of seedlings at nursery interferes on the final quality of the seedlings, which tend to root system folding and show low vegetative vigor for the constraint of exploitable space of the substrate. These situations were identified, from the fiftieth day, when despites the growth peaks, roots exceeded the drainage holes of the containers and the leaves started to become yellowish, this fact being an indicative of malnutrition (Minami, 2010).

Since no methodology to determine the ideal seedling transplant age (Mafia *et al*., 2005) can be found, the evaluation of root and shoot dry mass ratio (MMSSR/MMSPA) was adopted in this study, the greatest value obtained in this analysis ensuring stability and resistance to bending by wind in the field. In this sense, *L. alba* seedlings showed satisfactory quality at thirty days, when they showed greater value for MMSSR/MMSPA ratio (0.081). This ratio is considered efficient and safe, due to its unlikely variation, being adopted as one of the indices that determine the quality of forest seedlings (Gomes & Paiva, 2011). According to Alfenas *et al*. (2004), an imbalance in this ratio can cause undesirable plants, weakened, susceptible to pests and diseases.

Variables and analysis methods used characterized the best time for seedling planting, considering the maintenance of vegetative growth and attributes of the root system. Despite the ease of rooting and development of cuttings which *L. alba* plants present (Pimenta *et al.,* 2007), the results obtained in this study show that devices used in cutting can improve the final result of seedling production and establishment of *L. alba* plants for commercial crops. Rooting talc can be recommended to growers, replacing pure IBA, due to its better cost/benefit analysis. Therefore, we can recommend the preparation of *Lippia alba* seedlings by cuttings with 10 mg/L rooting powder/talc, in substrate with mineral manure, vermicompost, clayey texture and sand commercially known as 'medium sand', during 30 days.

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