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Growth and Physiological Response of *Azadirachta excelsa* (Jack) Jacobs Seedlings to Over-Top-Filling Treatment

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ABSTRACT

Over-top-filling is one of common stresses experienced by urban landscape trees. Flexibility of morphological and physiological is needed by trees to survive in over-top-filling. This study was conducted to determine the effect of over-top-filling on *Azadirachta excelsa* seedlings by imposing different levels of soil over-top-filling, i.e, 10, 20 and 30 cm. Soil was mounted above the normal collar and covered. Growth and physiological characteristics were assessed, and repeated measures analysis was used to analyze the differences among times and treatments. The repeated measures results showed various patterns of morphological growth throughout the experiment. In general, treated *A. excelsa* seedlings showed positive growth due to the extra availability of nutrients in the soil. Leaf mass ratio was high in the 30-cm over-top-filling treatment, indicating a large amount of chlorophyll. In addition, new development of root area showed a persistent relation to root sensitivity of the system architecture by increasing root volume. Chlorophyll fluorescence was found to be higher in the treated seedlings compared to the controls. Gas exchange attributes were also found to vary among treatments, but not other water-related parameters such as predawn and midday water potential, hydraulic conductance, and water use efficiency. It was decisively found that this species is partially tolerant of over-top-filling due to its ability to resist permanent damage and due to its stress avoidance.

Key words: *Azadirachta excelsa*, over-top-filling, repeated measures, biomass, chlorophyll fluorescence, gas exchange, hydraulic conductance

INTRODUCTION

In recent years, many studies and debates on the issues of tree protection have taken place among urban foresters, arborists and researchers. One of the hot topics in tree protection is in regard to the long-term risks of placing fill soil over a seedling's stem while it is growing. This practice, called over-top-filling, occurs when soil is mounted over the normal tree collar while the plant is growing (Day, 1999). It is similar to planting trees too deep into the soil but these two situations are different from the perspective of application. Whereas planting trees too deep into their planting holes is due to improper procedures on the part of planters during tree transfer into the planting area, over-top-filling occurs over a period of time and the soil eventually increases to overwhelm the normal collar. This scenario takes place mainly in development areas.

Using an excessive amount of soil may be caused by bad management practices, whether intentional or unintentional, that occur during nursery production and harvest, landscape installation, or landscape maintenance (Day and Harris, 2008; Rathjens, 2009). Another cause is mounding the soil higher than the collar in order to prevent the trunk from being in an unstable position in the early planting stages. In some cases, the condition is purposely created to provide proper drainage, to create gentle inclines for pedestrians and cars and sometimes, to meet certain engineering requirements. Smiley and Booth (2000) surveyed 417 trees in the eastern United States and found that 93% of the trees had soil and/or mulch over the root flare where soil generally exceeded not less than 5.0 cm. This condition also occurs due to natural causes, such as when loose soil rolling downhill during rainfall results in excessive soil accumulating over the root. If this happen slowly and frequently, the soil will accumulate around the basal areas of trees. Excessive soil can be removed easily from over-top-filled trees but as mentioned by Watson and Himelick (1997), when roots are planted too deeply, the situation cannot be corrected easily.

The effects of over-top-filling stress on trees are still not widely known. However, the consequences of this stress are equivalent to the effects of deep planting. Deeply planted structural roots can ruin tree establishment and stunt tree growth, shorten lifespan, cause nutrient deficiencies, increase stress, decrease stability and lead to the formation of stem girdling roots (Rathjens *et al.*, 2009). Other common problems that occur in landscaping management are related to the planting depth of the tree which can affect the root system. Researchers have shown that trees such as *Phoenix roebelennii* O'Brien, *Pinos strobus* L., *Prunus yedoensis* Matsum and *Koelreuteria bipinnata* Franch cannot survive well and experience a mortality rate of up to 100% if planted at 15 cm below girth (Arnold *et al.*, 2004; Broschat, 1995; Smiley and Booth, 2000; Wells *et al.*, 2006).

Additional potential damage from over-filling can result from direct contact of the trunk with the soil, as soil-borne diseases can be disseminated though the bark and lead to infection (Day and Harris, 2008). On the positive side, physical contact between soil and the tree bark triggers the production of girdling root formation, called epitomic root which specializes in obtaining nutrients and water. However, this root does not provide structural stability. A study on silver maple and flowering dogwood shows that formation of a new root system from the trunk is a sign of successful adaptation to an over-top-filling condition (Day, 1999). Some suggestions for treating seedlings that are too deeply planted involve replanting and root crown excavation (Rathjens *et al.*, 2009). Most studies typically recommend a shallow planting depth of 15 cm or less (Arnold *et al.*, 2005, 2007; Gilman and Grabosky, 2004).

The scope of studies on appropriate planting depth and their circumstances under unfavorable conditions is limited. Thus, this study was carried out to investigate the response of morphological characteristics, including relative growth of diameter, height and leaf area and physiological characteristics, including chlorophyll content, chlorophyll fluorescence, gas exchange, hydraulic conductance and water potential, to over-top-filling. It was hypothesized that as the height of soil increased above the normal collar against a seedling's stem, the greater would be the decline in the tree's performance.

MATERIALS AND METHODS

Study site: The experiment was conducted in the nursery (open area) of the Faculty of Forestry, Universiti Putra Malaysia, Serdang, Selangor, from the end of October 2011 until March 2012. The average daily temperature varied from about 27-30°C, the relatively humidity varied from 60-71%

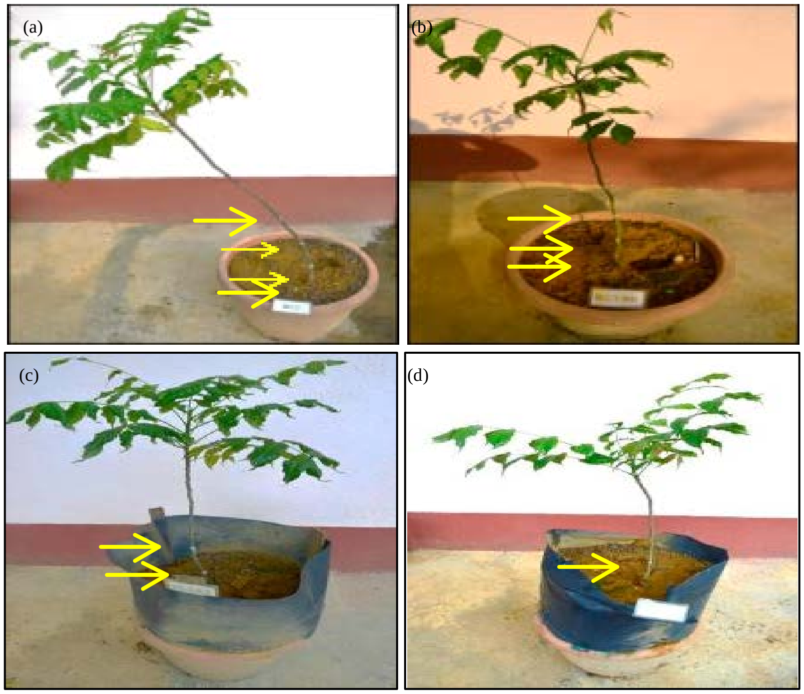

Fig. 1(a-d): Levels measured in over-top-filling experiment, (a) Control, (b) 10 cm over-top-filling, (c) 20 cm over-top-filling and (d) 30 cm over-top-filling

and the irradiation range was around 609.48 μM m^2 sec^{-1}, PAR as measured by a Watch Dog Model 2475 Plant Growth Station (Spectrum Technologies Inc., Aurora, IL).

Seedling preparation: Thirty-three healthy *A. excelsa* seedlings of the same age and from the same source were planted in pots with a top soil mixture of 3:2:1 soil:sand:peat at the normal collar (about 5 cm above the root growth area). The seedlings were acclimatized to the nursery conditions for two months. Acclimatization is important in order to avoid shock in the seedlings and bias during data collection. The seedlings were grown under natural sunlight conditions and were irrigated with a sprinkler daily, in the morning and evening. The seedlings were fertilized using Grofas Green foliar fertilizer (CCM Fertilizer, Malaysia) in rate of 10 g weekly.

Experimental design: The structure of the experiment was a Completely Random Design (CRD), wherein the pots were arranged randomly by using a number generator. At the end of the two month's acclimatization f seedling process, the over-top-filling treatment was applied by mounding soil above the normal girdling, at heights of 10, 20 and 30 cm from the normal root collar. The extra soil was not a mixture of soil, sand and peat but top soil only, to simulate real conditions in the field. In order to prevent the soil from overflowing the pots, custom-made polyethylene frames were constructed. The frames were made of plywood waste that was stapled to the polyethylene.

Data collection: In order to conduct a nondiscriminatory evaluation of seedling growth and assuming that the various parts of the seedlings experience different growth rates, the data collection was different at the different levels of treatment-10, 15, 25 and 35 cm as shown in Fig. 1.

Growth measurement: Growth data were taken for every first week of month. Seedling diameters were recorded with a digital vernier caliper (Mitutoyo UK Ltd., Hampshire, UK) 10 cm above the collar and heights were measured with a meter ruler.

The measurements support the data on growth increment throughout the experiment in terms of Absolute Growth Rate (AGR) and Relative Growth Rate (RGR). The AGR measurement includes the diameter and height growth using an average of three readings for each seedling. The average of the diameter or height was used to calculate the absolute growth rate, as follows:

$$AGR_{diameter}\,(mm\ week^{-1}) = \frac{D_2 - D_1\,(mm)}{T_2 - T_1\,(week)} \quad (1)$$

$$AFR_{height}\,(cm\ week^{-1}) = \frac{H_2 - H_1\,(cm)}{T_2 - T_1\,(week)} \quad (2)$$

where, $AGR_{diameter}$ is absolute diameter growth rate, AGR_{height} is absolute height growth rate, D_1/H_1 is diameter or height at T_1, D_2/H_2 is diameter or height at T_2, T_1/T_2 is time.

Relative Growth Rate (RGR) was calculated for a time period of linear growth as:

$$RGR = \frac{In\ D_2 - In\ D_1\,(mm)}{T_2 - T_1\,(week)} \quad (3)$$

Biomass from each part of the seedlings was measured as an absolute growth estimator. The samples of the tree components were oven-dried at 75°C until a constant weight was reached. The components consisted of aboveground biomass, determined by calculating the sum of the biomasses of shoots, leaves and stems. Other components were root mass as the belowground biomass and total seedling biomass calculated as the sum of the aboveground biomass and root biomass.

Measurement of leaf ecophysiological traits and leaf characteristics: Three fully expanded leaves from each individual seedling were selected as sample for measurement. The measurements were collected in the similar time of data collection, to minimize changes through months which may occur in leaf water content and irradiance level. The chlorophyll contents of the composite leaf samples from each treatment were determined using a SPAD 502 Plus chlorophyll meter (Spectrum Technologies Inc., Aurora, IL). The index of chlorophyll content was known as SPAD value.

Using the same leaves from the *A. excelsa* seedling, photochemical efficiency was measured using a Handy Pea chlorophyll fluorometer (Hansatech Instruments, Norfolk, UK), wherein dark leaf clips were applied to stop any photosynthetic process. After 15 min, PAR was supplied and data on initial fluorescence (F_o), maximum fluorescence (F_m) and the maximum photochemical efficiency of photosystem II (PSII) indicated by the ratio of variable to maximum fluorescence (F_v/F_m where, $F_v = F_m$-F_o) were recorded.

Leaf gas exchange rate was measured with a LI-6200 portable photosynthesis meter (LI-COR Inc, Lincoln, NE.). Data was taken in the morning, from 0800-1100 h, to avoid the state where they achieve optimum photosynthetic rate at midday (Marra and Heinemann, 1982). All measurements were taken at a flow rate of 250 μm sec^{-1}, reference CO_2 concentration of 360 μm CO_2 mol^{-1} (ppm) and 650 μm photons m^{-2} sec^{-1} of quantum flux. The responses were quantified by measuring the photosynthesis rate (A_{net}), stomatal conductance (G_s), intercellular CO_2 (C_i), transpiration rate (E) and leaf to air vapor pressure deficit (VpdL) calculated based on leaf temperature (D). The leaf samples was then picked to measure leaf area using a LI-3100 leaf area meter (LI-COR Inc., Lincoln, NE).

Whole seedling hydraulic conductance (K) and water use efficiency: Briefly, hydraulic conductance (K) determines water movement across the membrane (Hsiao, 1973; Wullschleger *et al*., 1998). The changes occur during hydraulic conductivity and exposure of the woody stem to xylem cavitation and the significance of water supplies is evaluated. Typically, the larger the K value, the greater is the flow rate. K calculations were performed based on measured water potential and whole seedling transpiration as:

$$K\,(\text{kg kPa}^{-1}\ \text{sec}^{-1}) = \frac{E}{-\psi p-(-\psi m)} \tag{4}$$

where, K_p is whole-seedling hydraulic conductance, E is transpiration rate, Ψ_p is predawn leaf water potential, Ψ_m is midday leaf water potential.

Whole seedling transpiration rate was measured by using a formula that uses data on weight loss over time:

$$E = \frac{W_2 - W_1\ (\text{kg})}{T_2 - T_1\ (\text{hour})} \tag{5}$$

Meanwhile, water potential (Ψ) was measured by taking one healthy leaf from each seedling, plucked at predawn and midday, to determine its water potential using a leaf pressure chamber (PMS Inc., Corvallis, OR).

Water Use Efficiency (WUE) can be classified into two types, intrinsic WUE (WUE_i) and instantaneous WUE (WUE_{inst}), according to the following equations derived from parameters gained from gas exchange measurements (Fischer and Turner, 1978):

$$WUE_i = \frac{A_{net}\ (\mu\text{mol } CO_2)}{g_S\ (\text{mol } H_2O)} \tag{6}$$

$$WUE_{inst} = \frac{A_{net}\ (\mu\text{mol } CO_2)}{E\ (\text{mmol } H_2O)} \tag{7}$$

Generally, WUE_i only focuses on the situations inside the stomata opening, while WUE_{inst} is influenced by the changing evaporative demands on water flux outside the leaf.

Data analysis: All analyses were performed using repeated measure Analysis of Variance (ANOVA) carried out with SPSS version 21.0 statistical software (SPSS, Chicago, IL) to identify differences between time, treatment and their interaction except for biomass. Curve fitting and graph handling were then performed using Sigma Plot version 10.0 (SPSS Inc., San Rafael, CA). All tests were considered significant at $p<0.05$.

RESULTS

Survival rate within treatment: Percentage of *A. excelsa* seedlings' survival at each treatment was recorded; the main effect of month on survival rate was significant ($F\ (6,\ 174) = 0.931$,

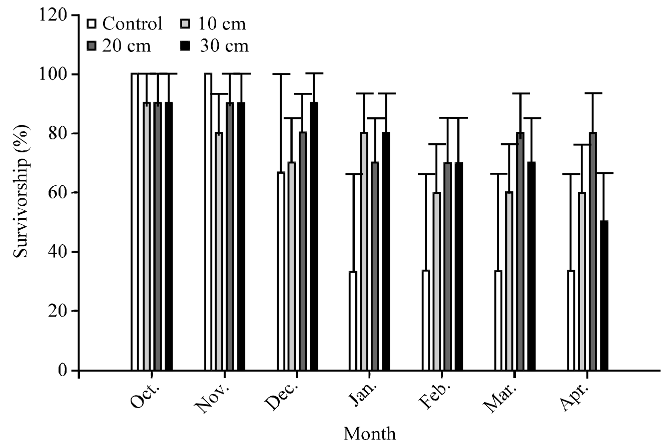

Fig. 2: Survival rate over time of *A. excelsa* seedlings planted at different levels of over-top-filling

Table 1: Repeated measures analysis within and between subjects of the effects of over-top-filling and time on absolute diameter growth rate ($AGR_{diameter}$) at different collar heights: 15, 25 and 35 cm

SOV	MS	df	F
$AGR_{diameter}$ at 15 cm			
Time	0.009	5,55	1.798^{ns}
Time*Treatment	0.065	5,55	1.176^{ns}
Treatment	0.009	1,11	0.042^{ns}
$AGR_{diameter}$ at 25 cm			
Time	0.122	5,100	4.550**
Time*Treatment	0.052	10,100	1.941^{ns}
Treatment	0.119	2,20	1.738^{ns}
$AGR_{diameter}$ at 35 cm			
Time	1.428	5,145	31.196***
Time*Treatment	0.072	15,145	1.576^{ns}
Treatment	0.176	3,29	0.497^{ns}

ns: Non-significant, **$p<0.01$, ***$p<0.001$

$p<0.001$), while no significant differences were found between treatments ($F(3, 29) = 1.422$, $p = 0.796$). It was observed that the control seedlings shown the highest survival rate from October to November 2012 but then decreased significantly to 60% and maintained that level until the end of the experiment (Fig. 2). The seedlings that underwent 10 cm over-top-filling treatment declined continuously from the first month, then increased at the third month before decreasing again in the following month. The seedlings with 20 cm over-top-filling showed great performance and recorded the highest survival percentage (80%) at the end of the experiment. The seedlings with 30 cm over-top-filling showed great performance compared to the control toward the end of the experiment.

Morphology performance within treatment: As mentioned previously, in order to gain reliable data comparisons among levels for each treatment, diameter measurements were taken at 10 (control), 15 (10 cm), 25 (20 cm) and 35 (30 cm) cm. The results showed that there were no significant differences between months for the 15 cm collar height when a repeated measures ANOVA analysis with a Greenhouse-Geisser correction was performed. However, mean $AGR_{diameter}$ at collar heights of 25 and 35 cm showed significant differences across time (Table 1, Fig. 3).

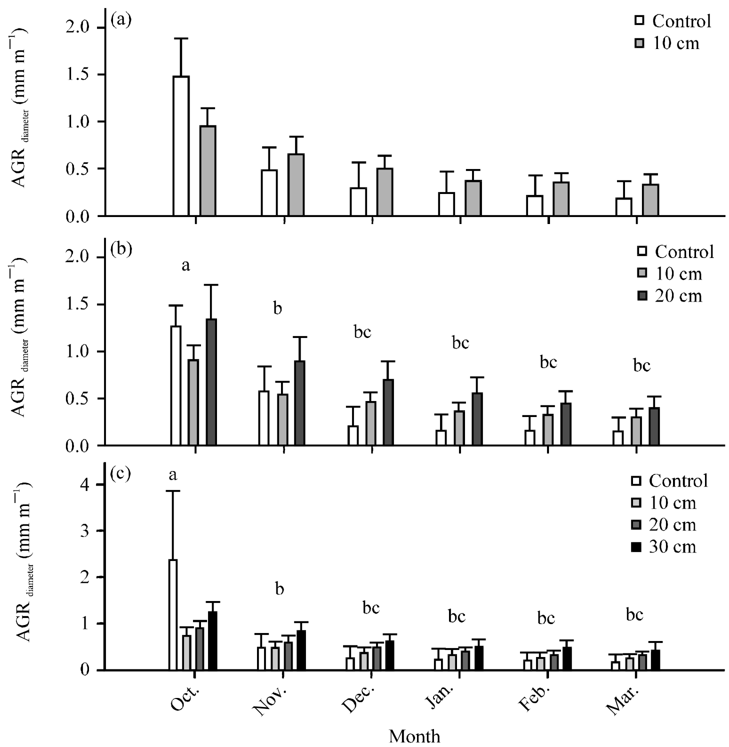

Fig. 3(a-c): $AGR_{diameter}$ over time of *A. excelsa* seedlings planted at different over-top-filling levels, Data were taken at collar heights of (a) 15 cm, (b) 25 cm and (c) 35 cm, Data are the mean of each treatment replicates±standard deviation, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

Moreover, analysis of between-subjects effects showed that there were no significant differences among treatments at all collar heights.

$AGR_{diameter}$ of *A. excelsa* trended high in the 10 cm over-top-filling treatment in the final two months, at 0.2715±0.1012 and 0.1735±0.0856 mm (Fig. 3). Throughout the experiments, a significant effect on diameter increment of the seedlings was seen in the first month, specifically for treatment levels 20 and 30 cm; however, the seedlings showed decreased diameter increment in the following month. In addition, no absolute increment in diameter occurred with the 10 cm over-top-filling compared to the control condition. However, there were slight diameter increments in the 20 and 30 cm treatments, where in the final two months, the $AGR_{diameter}$ of the treated seedlings were higher than that of the controls when seedlings were mounded with over 10 and 20 cm of top soil (Fig. 3).

There were also significant differences in seedling $RGR_{diameter}$ throughout the experimental period for data taken at the 15, 25 and 35 cm collar levels. In addition, there were no significant results at all collar heights when compared between treatments (Table 2). As seen in the pattern shown in Fig. 4, at all levels of treatment, there was a significantly higher increment in diameter in the first month than in the other months. By the third month, the seedlings showed a significant decrease in diameter increment. As with $AGR_{diameter}$, it can be seen that the seedlings in 20 and 30 cm had higher diameter increments compared to the controls but the differences were not significant (Fig. 4).

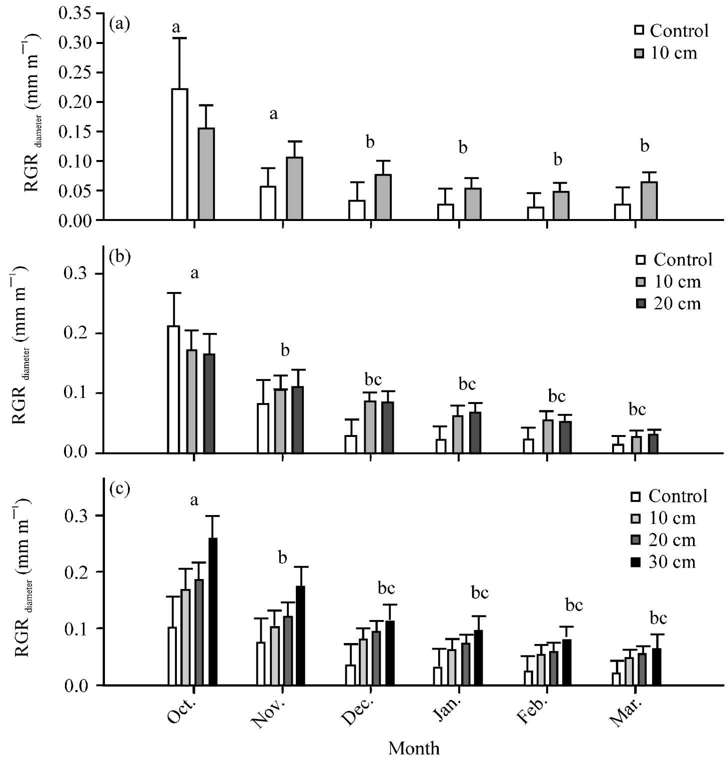

Fig. 4(a-c): $RGR_{diameter}$ over time of *A. excelsa* seedlings planted at different over-top-filling levels, Data were taken at collar heights of (a) 15 cm, (b) 25 cm and (c) 35 cm, Data are the mean of each treatment replicates±standard deviation, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

Table 2: Repeated measures analysis within and between subjects of the effects of soil bulk density and time on relative diameter growth rate ($RGR_{diameter}$) at different collar heights: 15, 25 and 35 cm

SOV	MS	df	F
$RGR_{diameter}$ at 15 cm			
Time	0.119	5,55	12.987***
Time*Treatment	0.017	5,55	1.884^{ns}
Treatment	0.012	1,11	0.317^{ns}
$RGR_{diameter}$ at 25 cm			
Time	0.168	5,100	31.708***
Time*Treatment	0.005	10,100	1.306^{ns}
Treatment	0.007	2,20	0.206^{ns}
$RGR_{diameter}$ at 35 cm			
Time	0.205	5,145	34.135***
Time*Treatment	0.005	15,145	0.787^{ns}
Treatment	0.049	3,29	1.157^{ns}

ns: Non-significant, ***p<0.001

Regarding height characteristics, repeated measures ANOVA indicated that time had a significant effect on all data taken at 15, 25 and 35 cm for AGR_{height} (Table 3). In contrast, when

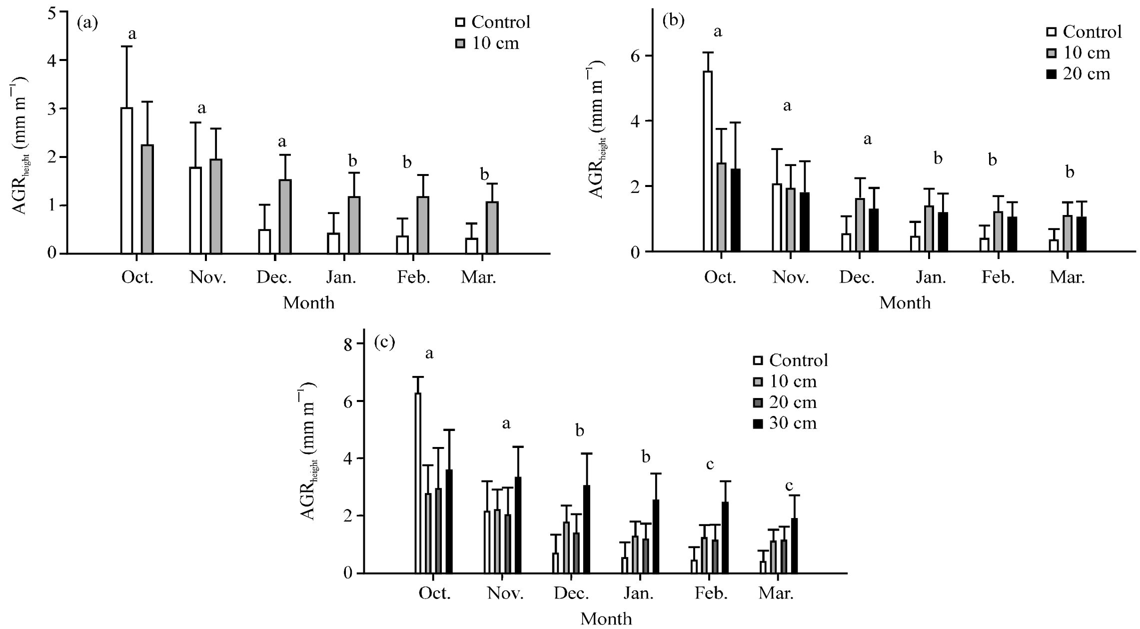

Fig. 5(a-c): AGR$_{height}$ over time of *A. excelsa* seedlings planting at different over-top-filling levels, Data were taken at collar heights of (a) 15 cm, (b) 25 cm and (c) 35 cm, Data are the mean of each treatment replicates±standard deviation, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

Table 3: Repeated measures analysis within and between subjects of the effects of soil bulk density and time on absolute height growth rate (AGR$_{height}$) at different collar heights: 15, 25 and 35 cm

SOV	MS	df	F
AGR$_{height}$ at 15 cm			
Time	1.082	5,55	9.430**
Time*Treatment	0.374	5,55	3.264^{ns}
Treatment	0.315	1,11	0.242^{ns}
AGR$_{height}$ at 25 cm			
Time	2.084	5,100	18.536***
Time*Treatment	3.433	10,100	4.694**
Treatment	0.112	2,20	0.079^{ns}
AGR$_{height}$ at 35 cm			
Time	2.405	5,145	15.328***
Time*Treatment	0.28	15,145	2.098^{ns}
Treatment	0.481	3,29	0.341^{ns}

ns = non-significant, **p<0.01, ***p<0.001

comparisons were made between treatments, no significant differences were found at all collar heights. The trend in AGR$_{height}$ over the course of the experiment is shown in Fig. 5, generally, height increment declined significantly beginning in January 2012 (fourth month of the experiment). However, there was an increment in height in the10 cm over-top-filling seedlings

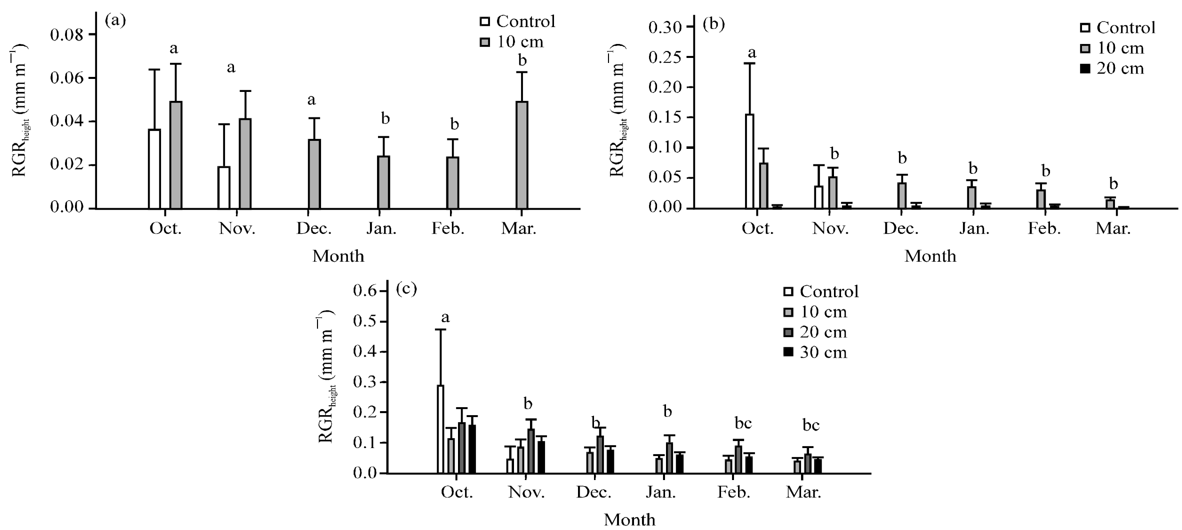

Fig. 6(a-c): RGR_{height} over time of *A. excelsa* seedlings planted at different over-top-filling levels, Data were taken at collar heights of (a) 15 cm, (b) 25 cm and (c) 35 cm, Data are the mean of each treatment replicates±standard deviation, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

Table 4: Repeated measures analysis within and between subjects of the effects of over-top-filling and time on relative height growth rate (RGR_{height}) at different collar heights: 15, 25 and 35 cm

SOV	MS	df	F
RGR_{height} at 15 cm			
Time	0.006	5,55	3.919*
Time*Treatment	0.002	5,55	1.160^{ns}
Treatment	0.023	1,11	1.736^{ns}
RGR_{height} at 25 cm			
Time	0.092	5,100	13.354***
Time*Treatment	0.014	10,100	1.840^{ns}
Treatment	0.003	2,20	0.108^{ns}
RGR_{height} at 35 cm			
Time	0.014	5,145	4.549**
Time*Treatment	7.170	15,145	1.164^{ns}
Treatment	0.003	3,29	0.677^{ns}

ns: Non-significant, *p<0.05, **p<0.01, ***p<0.001

compared to the controls. Positive effects were also seen at the 25 and 35 cm collar heights, in that the seedlings showed higher increments than the controls in almost all readings, supported by the interaction results.

The RGR_{height} results indicated that *A. excelsa* responded differently over time at all collar heights (Table 4). Above all, the between-subjects effects of treatments suggested that no treatment was significantly different from the others at all collar heights. As shown in Fig. 6, higher actual increments occurred in the treated seedlings than in the controls. However, the seedlings planted at treatment levels of 20 and 30 cm showed a significant decline in height increment in the second month.

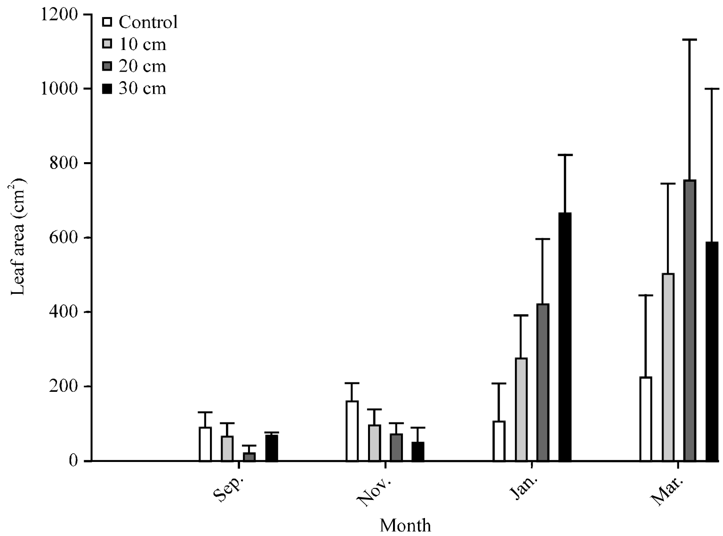

Fig. 7: Leaf area per month of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM shown by vertical error bars

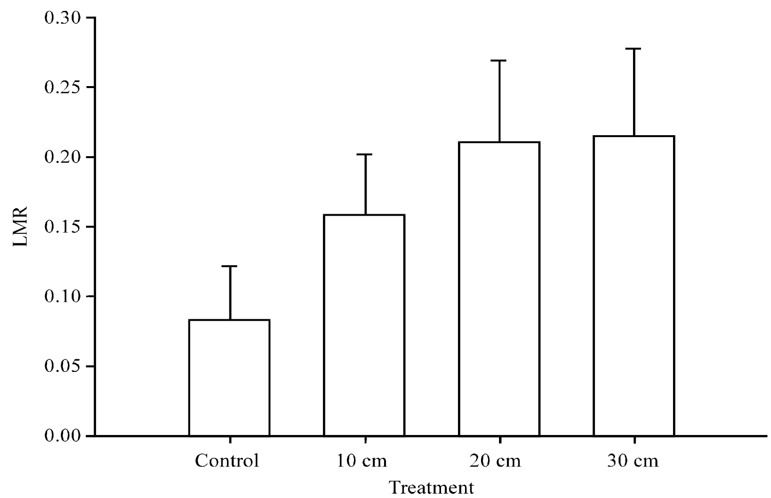

Fig. 8: Leaf Mass Ratio (LMR) of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM, shown by vertical error bars

Leaf area adaptation: The results show that there were no significant effects of over-top-filling on the leaf area over time (F (5, 145) = 1.599, p = 0.217) or between treatments (F (3, 29) = 0.191, p = 0.91). However, the leaf area results showed that all of the treated seedlings had lower leaf areas in order to adapt to the conditions during the first two months, while over the last two months, higher leaf areas were recorded at the higher over-top-filling levels (Fig. 7). Further analysis showed that leaf mass ratio associated with high RGR indicated that a high allocation of leaf mass appeared in the seedlings treated at the higher levels (Fig. 8).

Biomass allocation: One-way ANOVA indicated no significant differences in the biomass of *A. excelsa* among the different over-top-filling treatments for all plant components (Table 5).

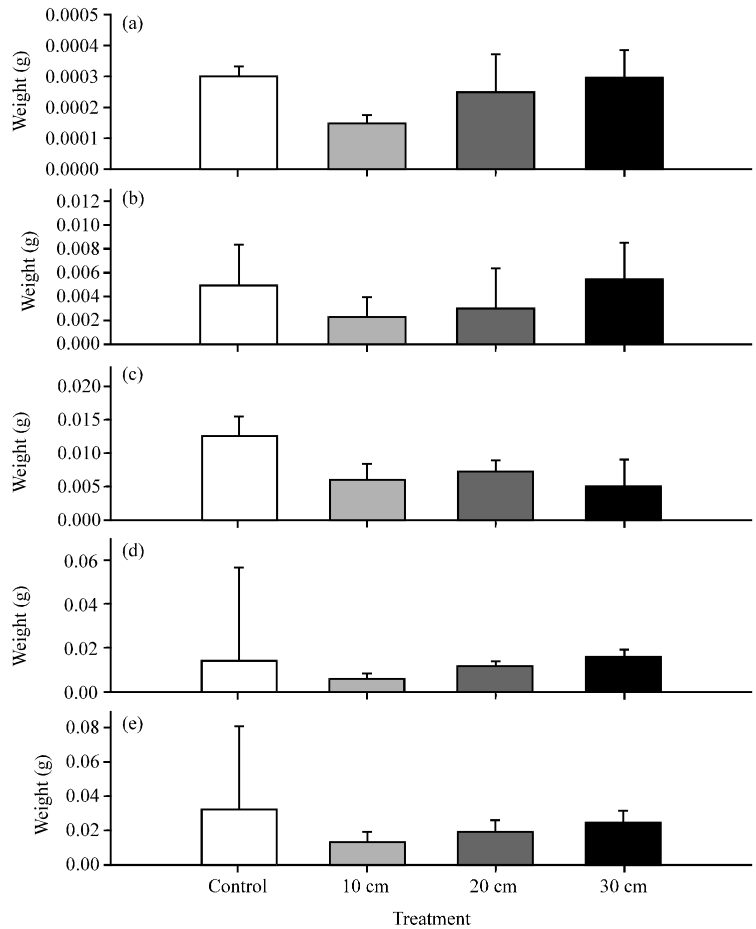

Fig. 9(a-e): Biomass of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM, shown by vertical error bars, (a) Shoot dry weight, (b) Leaves dry weight, (c) Stem dry weight, (d) Root dry weight and (e) Total dry weight

Table 5: ANOVA of effects of over-top-filling treatment on each part of the seedling biomass, including shoot, leaf, stem, root and total biomass

Effects (g)	df	F
Shoot biomass	3,17	0.674^{ns}
Leaf biomass	3,17	0.185^{ns}
Stem biomass	3,17	0.957^{ns}
Root biomass	3,17	2.047^{ns}
Total biomass	3,17	1.823^{ns}

ns: Non-significant

Generally, the biomass components, including shoots, leaves and roots, increased the most at the 30 cm over-top-filling level compared to the 10 cm level (Fig. 9).

It was also observed that *A. excelsa* seedlings tend to adapt by producing new areas of roots along the stem that was covered by over-top-filling (Fig. 10).

Further analysis showed that although root-to-shoot ratio (RSR) was not significant ($F (3, 17) = 0.394$, $p>0.05$), changes occurred in signaling carbon allocation (Fig. 11).

Fig. 10: New root system along the buried stem

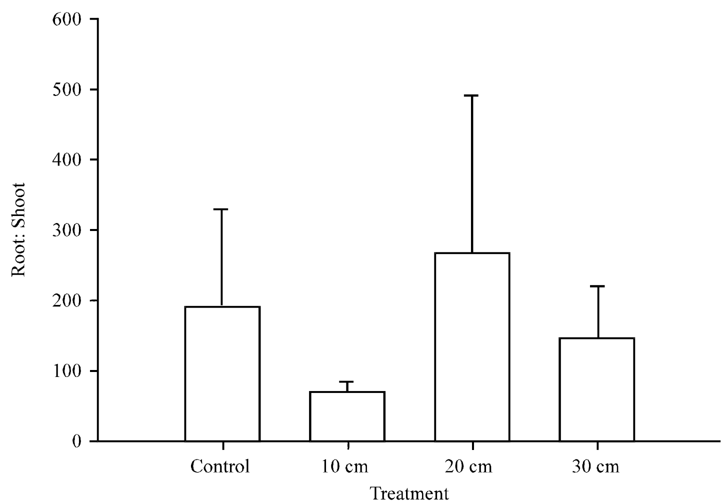

Fig. 11: Root-to-shoot ratio of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM, shown by vertical error bars

Comparatively, there was an increase in RSR at the 20 cm over-top-filling level which indicates a high capability of producing more roots, rather than growing shoots.

Leaf physiological traits: Highly significant differences in chlorophyll content were found across the experiment ($F (6, 174) = 11.539$, $p<0.001$) but no differences were found between treatments ($F (3, 29) = 0.445$, $p = 0.723$). All treated seedlings achieved a higher amount of chlorophyll content compared to the controls two months after planting and continuing until the end of the experiment (Fig. 12).

A repeated measures ANOVA with a Greenhouse-Geisser correction determined that the means of all chlorophyll fluorescence parameters differed significantly among months (Table 6). Analysis

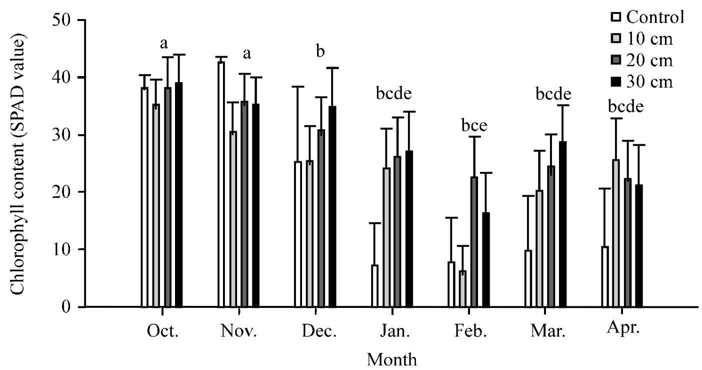

Fig. 12: Chlorophyll content using SPAD value per month of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM., shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

Table 6: Repeated measures ANOVA within and between subjects of the effects of over-top-filling and time on the chlorophyll fluorescence parameters F_o, F_m, F_v, and F_v/F_m

SOV	MS	df	F
Fo			
Time	3849958.940	6,174	4.720***
Time*Treatment	923185.246	18,174	1.132^{ns}
Treatment	2478721.109	3,29	0.452^{ns}
Fm			
Time	111599376.495	6,174	9.362***
Time*Treatment	6281172.422	18,174	0.527^{ns}
Treatment	89888132.142	3,29	1.148^{ns}
Fv			
Time	74960643.686	6,174	11.494***
Time*Treatment	5575545.834	18,174	0.855^{ns}
Treatment	15557131.050	3,29	0.327^{ns}
Fv/Fm			
Time	0.479	6,174	8.638***
Time*Treatment	0.044	18,174	0.795^{ns}
Treatment	0.217	3,29	0.468^{ns}

Note: ns: Non-significant, ***p<0.001

of time and treatment interactions and between treatments showed no significant differences. From January until the end of the experiment, there were significant decreases in all parameters except Fo which increased in March (Fig. 13). It is known that the optimal value for chlorophyll fluorescence in a tree in a good condition is in range 0.83. At the end of this experiment, the control seedlings displayed an average of 0.69 which indicated that all of the seedlings in this experiment could be physiologically stressed. The 10, 20 and 30 cm over-top-filling seedlings displayed levels of 0.72, 0.70 and 0.75, respectively.

The repeated measures ANOVA revealed significant differences among months for over top filling in terms of net photosynthesis (A_{net}), intercellular CO_2 (C_i), transpiration rate (E) and leaf-to-vapor pressure deficit in leaves (VpdL). It was shown in Fig. 14 for all treatments, the gas exchange parameters was found decreased significantly starting in second or third month which

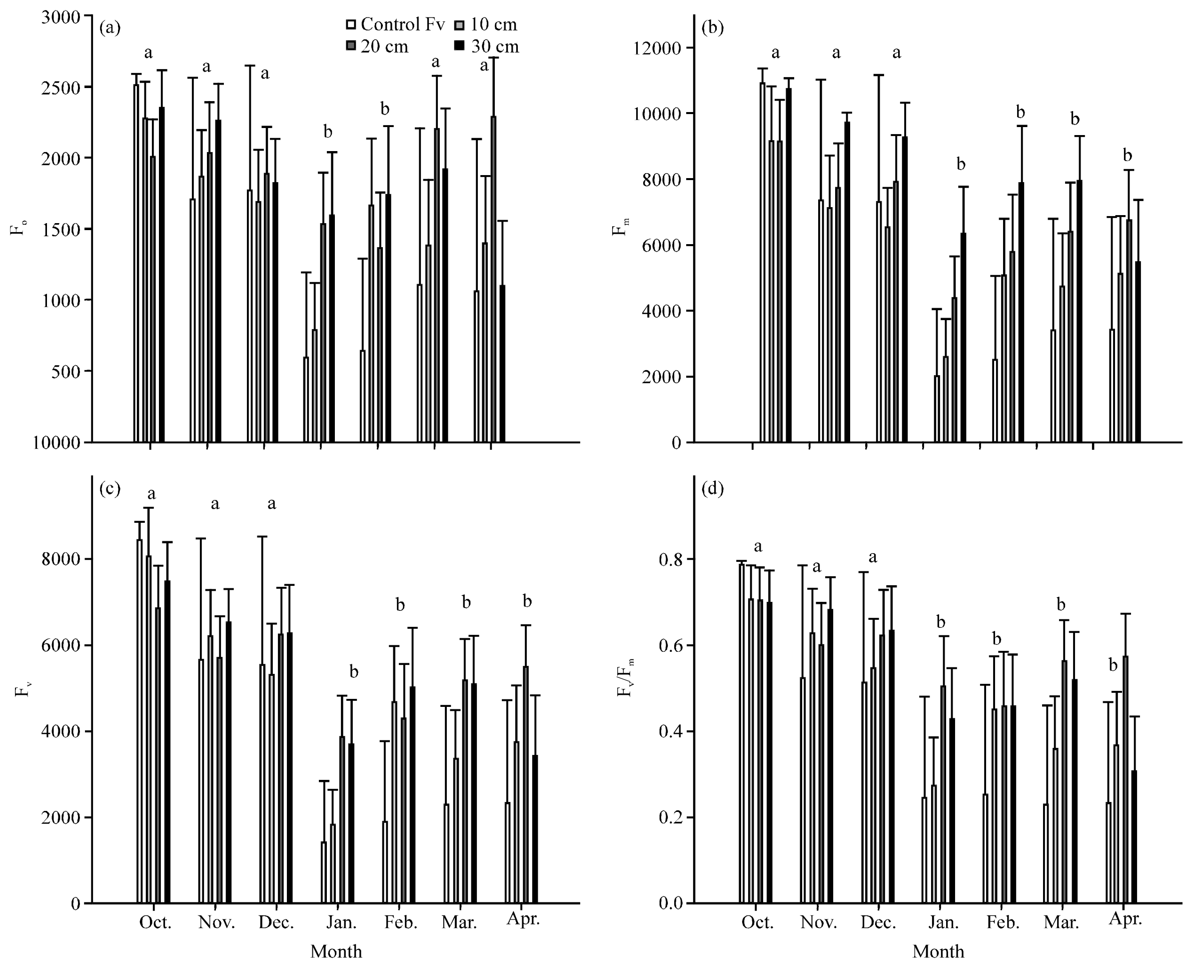

Fig. 13(a-d): Mean values over time of chlorophyll fluorescence parameters (a) F_o, (b) F_m, (c) F_v and (d) F_v/F_m of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

then maintain until end of experiment. While no significant differences in all gas exchange parameter were observed between treatments and time and treament interactions (Table 7).

Hydraulic conductance and water potential: There were no significant differences in hydraulic conductance by treatment and time (F (3,5) = 0.593, p = 0.652 and F (2,10) = 2.843, p = 0.542, respectively). This indicates that over-top-filling treatment does not affect hydraulic conductivity in *A. excelsa* seedlings. However, it can be seen in Fig. 15 at 20 cm over-top-filling had large value of K.

There was a significant decrease in water potential over time for both leaf water potential at predawn and midday (Ψ) (F (2,10) = 2.346, p = 0.014 and F (2,10) = 3.376, p = 0.024, respectively). For all treatments, data was significantly changes in the fourth month of experiment (Fig. 16).

Table 7: Repeated measures ANOVA within and between subjects of the effects of over-top-filling and time on the gas exchange parameters for each treatment: Net photosynthesis (A_{net}), stomatal conductance (G_s), intercellular CO_2 (Ci), transpiration rate (E) and leaf-to-vapor pressure deficit in leaves (VpdL)

SOV	MS	df	F
A_{net}			
Time	24.153	6,174	3.858**
Time*Treatment	3.481	18,174	0.556^{ns}
Treatment	3.650	3,29	0.119^{ns}
G_s			
Time	0.049	6,174	0.813^{ns}
Time*Treatment	0.130	18,174	2.157^{ns}
Treatment	0.060	3,29	0.955^{ns}
C_i			
Time	183967.133	6,174	8.898***
Time*Treatment	13251.591	18,174	0.647^{ns}
Treatment	15463.583	3,29	0.258^{ns}
E			
Time	7.393	6,174	3.89*
Time*Treatment	1.234	18,174	0.649^{ns}
Treatment	0.421	3,29	0.152^{ns}
VpdL			
Time	6.686	6,174	6.465**
Time*Treatment	0.166	18,174	0.161^{ns}
Treatment	0.077	3,29	0.059^{ns}

ns: Non-significant, *p<0.05, **p<0.01, ***p<0.001

Water use efficiency: There were significant differences, between time in both intrinsic and extrinsic Water Use Efficiency (WUE) F (6,54) = 3.582, p<0.01 and F (6,54) = 5.976, p<0.001, respectively) (Fig. 17). These parameters were not affected at all to over top filling, due to the favourable conditions of net photosynthesis and transpiration. Changes of variation in this parameter were signs of the ability of *A. excelsa* to be flexible and well adapted to the stress of climate conditions.

DISCUSSION

Survival adaptation capability: At the beginning of the experiment, *A. excelsa* seedlings were shocked with an accumulation of soil over their normal collars after a two-month acclimatization period after planting at normal collar. The survival rates of the seedlings were observed. Eventually, the seedlings were capable of recovering and adapted to over-top soil by showing promising positive results compared with the controls. Similar result were found in deep planting of *Pinus elliottii* Engelm seedlings, where the survival rate increased (South *et al*., 2012). In addition, seedlings of *Pinus taeda* planted with half the stem buried showed a 95% survival rate in well-drained clay soil but only a 70% survival in poorly drained clay and 30% in poorly drained silt (Switzer, 1960). Browne and Tilt (1992) showed that *Acer rubrum* can survive in deep planting at 5 and 10 cm. Thus, it can be inferred that some species accomplish the best growth when there is soil over the seedling's normal collar height.

Effects on morphology: The significantly higher increments in diameter in the first month were due to the high availability of soil nutrients inside the extra soil region. This is due to excessive soil

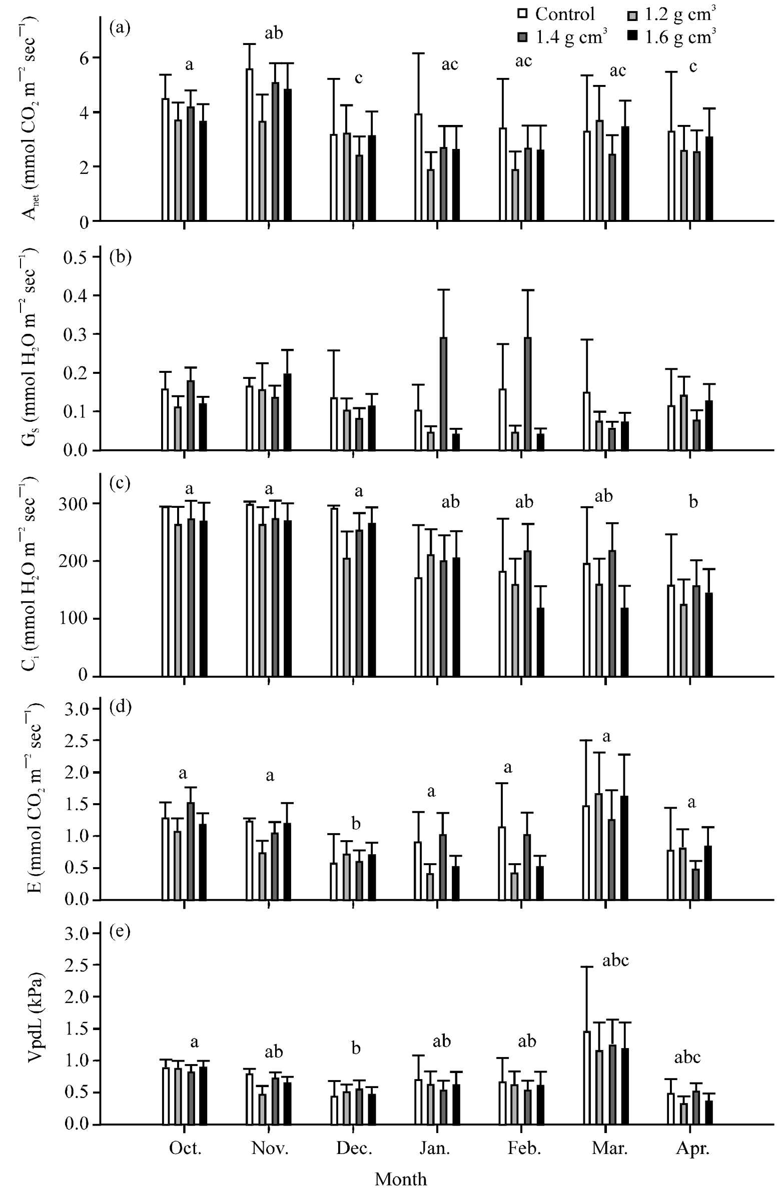

Fig. 14(a-e): Mean values over time of gas exchange parameters, (a) Net photosynthesis (A_{net}), (b) Stomatal conductance (G_s), (c) Intercellular CO_2 (C_i), (d) transpiration rate (E) and (e) leaf-to-vapor pressure deficit in leaves (VpdL)-for each treatment of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±standard deviation, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

which gives more space for the root to move around and extra availability of nutrients, allowing the seedling to experience healthy growth. Nutrient interception by growing roots helps seedlings grow in diameter as food translocation occurs. Unfortunately, in the long run, the soil eventually ran out of nutrients due to pot limitation which explains the significant decrease beginning the

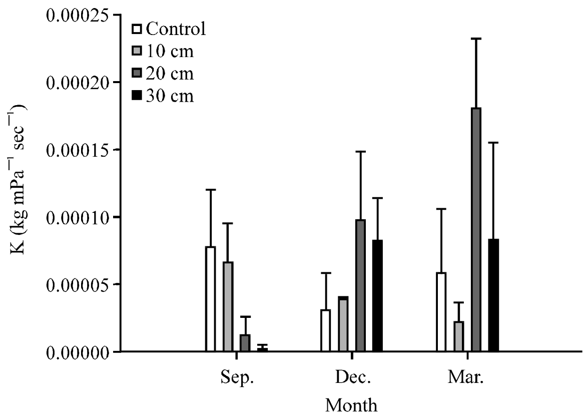

Fig. 15: Mean value over time of hydraulic conductance (K) of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM, shown by vertical error bars

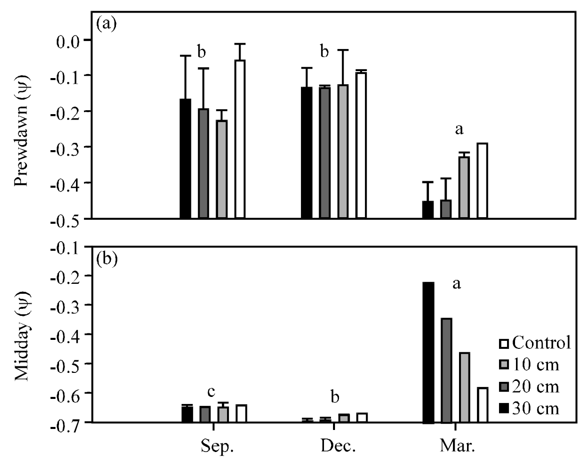

Fig. 16(a-b): Mean value over time of leaf water potential at (a) Predawn and (b) Midday (Ψ) of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

third month after planting. Similar results were also found in other deep planting studies of treatments by Rathjens (2009) and Wells *et al.* (2006). Notably, deep planting also provides the best growth performance in diameter, as well as normal planting. In general, no changes or disruption in diameter growth occurred which indicates an optimistic development for seedlings. However, study by Browne and Tilt (1992) found that diameter growth rate declined when *Cornus florida* was planted in deeper soil which is in unlike with the current results.

A very positive performance in trend of height increment of RGR_{height} was observed in all treatments; for instance, seedlings with 10 cm over-top-filling treatment had higher RGR_{height} values compared to the controls. It is believed that extra nutrient availability contributed to the seedlings' growth. Another study showed that *Malus* sp., had a positive response in tree height when its main lateral roots were planted deeply, 30 cm below the soil surface, compared to 0 and 15 cm, through

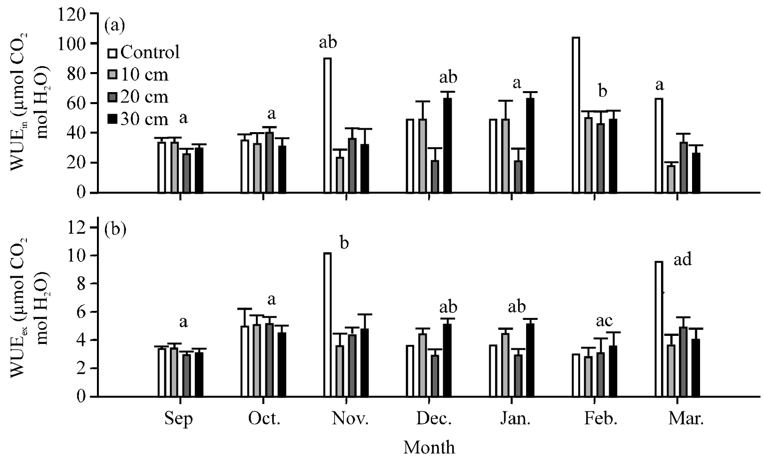

Fig. 17(a-b): Mean value over time of (a) Intrinsic and (b) Extrinsic Water Use Efficiency (WUE) of *A. excelsa* seedlings planted at different over-top-filling levels, Data are the mean of each treatment replicates±SEM, shown by vertical error bars, Lowercase letters indicate significant differences for all means comparisons with pairwise comparisons within each month

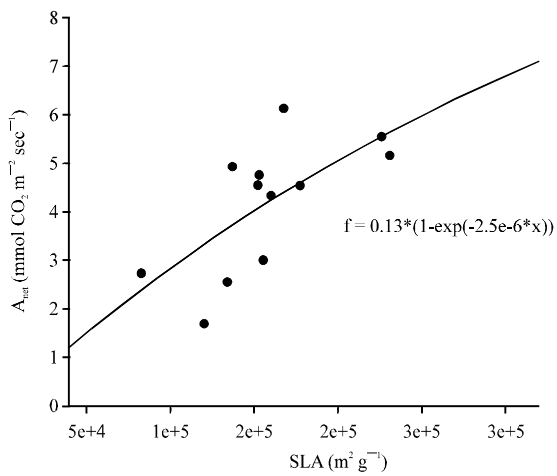

Fig. 18: Relationship between Specific Leaf Area (SLA) and net photosynthesis (A_{net})

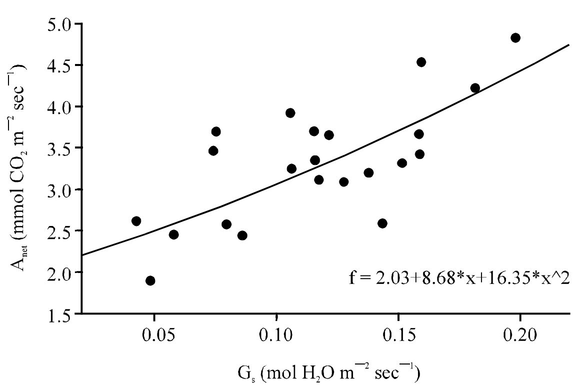

Fig. 19: Relationship between net photosynthesis (A_{net}) and stomatal conductance (G_s) in over-top-filling experiment

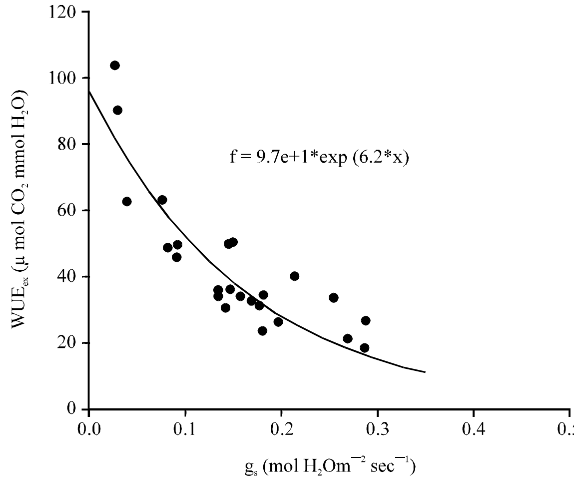

Fig. 20: Relationship between stomatal conductance and water use efficiency in over-top-filling

three years of planting (Rathjens, 2009). In addition, *Malus domestica* achieved greater height but fewer blooms when planted 5 cm below normal depth (Lyons *et al.*, 1987). The higher soil provided more nutrients for the seedlings to uptake. Thus, it can be emphasized that seedlings experience efficient growth due to the availability of more nutrients. Conversely, other study found that growth inhibition in some deeply planted trees is caused by a propensity of the roots being round all the soil or root distorted occurred (Seiler *et al.*, 1990). It can be concluded that the effects shown in over-top-filling studies were not significantly similar to those of planting depth studies (Browne and Tilt, 1992; Fare, 2005; Rathjens, 2009).

Biomass allocation: It was also observed that *A. excelsa* seedlings tend to adapt by producing new root areas along the stems that were covered by over-top-filling. This adaptation took place to solve the problem of oxygen deficit and extreme moisture (Gilman and Grabosky, 2004; Lloyd, 1997). It is also believed that the formation of the girdling roots, or epitomic roots, is to assist in obtaining nutrients and water. This root, however, does not provide structural stability without harm (Day and Harris, 2008).

The leaf area results indicate that after adaptation to top-filling, seedlings tend to grow larger leaves to produce better net assimilates aimed at higher productivity (Lambers *et al.*, 1998). The extra nutrients in the top-filling soil seemed to afford advantages to the seedlings by producing healthier leaves. In fact, it shows the role of chloroplast as a major component of the mass in the cytoplasm of mesophyll cells.

Seedlings in the treated soil levels seemed to allocate carbon as well as the control seedlings did. This characteristic is also supported by Specific Leaf Area (SLA), wherein higher SLA could be associated with higher net photosynthesis levels ($r = 0.6845$, $p<0.05$), as shown in Fig. 18. Similar results were found in the Gilman and Harchick (2008) study which reported the presence of trunk flare and visible surface roots in *Quercus virginiana* with increasing planting depth. Similar observations were found when a similar treatment was used in *Picea glauca* and *Acer rubrum* (Gilman and Kane, 1990; Sutton, 1967).

It was believed that the insignificant result in biomass parameters was caused by the age of the seedlings studied and the limitation of growing in pot conditions. If the experiment were extended to field conditions, there might be significant results in root development. Likewise, the over-top-filling did not influence the stress experienced by *A. excelsa*, indicating that cellular and chlorophyll metabolic activities were not affected at all by this type of stress (Taiz and Zeiger, 2010). This encouraging result was also found in *Malus* sp. which recorded a higher chlorophyll content at a top-filling depth of 30 cm (Rathjens, 2009). As discussed earlier in the leaf area results, Leaf Mass Ratio (LMR) and leaf biomass contributed to healthy leaf growth due to the additional nutrients in the over-top-filling soil. Macronutrients such as nitrogen are highly related to metabolic chlorophyll and may contribute to this phenomenon, as well as water availability at the surface in pot experiments.

Physiological toleration rates in leaves: The result in this experiment were different from the results of Wells *et al.* (2006), who found lower chlorophyll content at the leaf collar at a 30 cm planting depth compared to controls and a 15 cm depth. However, similar results were found in *Acer x freemanii* which showed the lowest chlorophyll content at a depth of 30 cm (Rathjens, 2009).

The photochemical efficiency values indicated that the ratio of absorbed energy used in photochemistry was not relatively affected by treatment (Jones, 1991). Repeated measures results indicated that the acceptance variation that occurred throughout the experiment was due to the ability of *A. excelsa* to adapt to the stress. Efficient photochemical and other physiological activities were also considered. Similarly, *Acer x freemanii* was found to have higher photochemical efficiency in shallower root systems (Rathjens, 2009). In addition, four years after planting, *Malus* sp., showed greater chlorophyll fluorescence at 30 cm top-filling than at 15 cm (Rathjens, 2009). As with chlorophyll readings, higher fluorescence was obtained with higher over-top soil which also proved that the seedlings were healthier.

It is known that carbon assimilation rates and water uptake can vary within genotype and gender levels and between species and that they affect gas exchange in larger spatial scales. This serves as the entry point for energy in a tree. Over-top-filling stimulated growth rate and increased the capacity to assimilate CO_2. The gas exchange parameters were not significantly different among treatments, indicating that mesophyll cells are not irreversibly affected by over-top-filling (Suresh *et al.*, 2010). Even in G_s, no significant result was found but there was a strong relationship between A_{net} and G_s in the over-top-filling experiment ($r = 0.7528$, $p<0.001$) (Fig. 19). The increase in A_{net} with over-top-filling was strongly correlated with the increase in G_s, suggesting that the changes in G_s were important in regulating A_{net} (Bejaoui *et al.*, 2012; Quentin *et al.*, 2012).

Water relation affect: The extra soil from over-top-filling may have a higher water holding capacity, thereby maximizing stomatal conductance and thus, transpiration. Briefly, it can probably be stated that these parameters indicate positive growth potential in *A. excelsa*. Significant decreases in water potential over time indicated that there was a substantial decrease in water flow in the seedlings in critical water stress at midday. Consequently, the adjustment of the *A. excelsa* seedling was helped by the formation of a new root system (Rathjens, 2009). It can also be suggested that top soil contains more moisture associated with water transport.

Further analysis found a significant relationship between stomatal conductance and water use efficiency ($r = 0.89$, $p<0.0001$) (Fig. 20). In the condition of no decreasing effect in other parameters, stomatal closure tends to increase the WUE_{in}. This is due to an optimum amount of carbon dioxide in leaf intercellular air spaces and favorable water pressure which relieves the condition for leaves wherein fewer stomata openings can produce high carbon assimilation.

CONCLUSION

As observed in this experiment, initial growth showed better results at the very beginning of the treatment, when the seedlings were found to grow well immediately, than when the soil was mounded above the collar. In the later stages, the seedlings were also found to adapt well to higher over-top-filling treatments. Other reactions of the seedlings found in this study were the maximization of the leaf area to help them tolerate the stress that was imposed. This step of adaptation purposely produced a high amount of chlorophyll and showed better seedling growth results than the controls. All of the treated seedlings showed better photochemical efficiency results than the controls, indicating that more electrons were captured in a shorter period of time and less energy was wasted. Positive results were also found in the gas exchange parameter which was shown to encourage growth rate and the capacity to assimilate carbon dioxide. In addition, no stress occurred in the seedlings in terms of water-related parameters, including hydraulic conductance, water potential and water use efficiency.

The most interesting finding was the discovery of a new root development area under the over-top-filling soil. This finding showed how the seedlings coped with the stress by taking advantage and obtaining nutrients within the area of the mounded soil. However, through repeated measures analysis, it was found that conducting the experiment in pots was not really suitable for studying the physiological performance of a woody tree. Using pots was a limiting factor for the seedlings to display actual morphology performance which in the study yielded non-significant differences. On the other hand, the leaves showed tremendous performance by significantly increasing the leaf area and exhibiting a high chlorophyll content and a strong relationship with high carbon assimilation. The seedlings also displayed good water-related performance even in higher treatments. This type of stress compels *A. excelsa* to develop adaptations that encourage growth.

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