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Full Length Research Paper

Effect of different substrates on survival and growth of transplanted orchids (*Dendrobium nobile* cv.) into net house

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Orchids are considered the most beautiful and valuable cut flower and potted plants all over the world. Although, micropropagation has become very important nowadays to meet growing market demand, there are still some barriers that hamper the ultimate goal to achieve viable ex vitro plants. Acclimatization has remained very serious issue. The current study was carried out to investigate the impact of different substrates available in nature on the survivability and development of ex vitro plantlets under net house conditions. The step-wise protocol of acclimatization utilized plantlets with well-developed roots produced in MS rooting medium involved NAA at 3.0 mg l^{-1}. The substrates used for acclimatization were different mixtures of coco-peat, desert sand, bricks chips, saw dust, coal pieces and date palm chips. The treatment contained coco-peat: desert sand: perlite at ratio 1.0:1.0:0.2 (v/v/v) proved the best mixture with 100% survival rate after 120 days of transplantation. Furthermore, addition of wood and bark chips to this best soil substrate as an upper layer improved the subsequent growth and development of plants. Number of new shoots and other growth parameters were recorded and the whole protocol is discussed in the current study.

Keywords: Acclimatization, coco-peat, hill sand, micropropagation, perlite, substrates.

INTRODUCTION

Orchids have an old history in terms of exploitation for therapeutic & ornamental purposes. They are considered as the most beautiful in ornamental plants due to their diverse shapes, colors, durable flowers and adaptability to room conditions. Their modern uses as cut flowers and potted plants have increased their demands in cut flower industries throughout the world. They account for 27% of the global cut flower production in terms of value (Kabiret al., 2012). The increasingdemand of orchid cut flowers and potted plants all over the world on different occasions has enticed the growers to adopt micropropagation technique to reach the market demands.

The micropropagation technique has been generally used worldwide for rapid and disease free propagation of several plant species but its broader use is restricted often by high percentage of morality or damage to plantlets during acclimatization (Deb and Imchen, 2010). This is a crucial step in micropropagation and is considered as difficult due to the variability of environmental factors from in vitro to ex vitro conditions. Generally, the conditions during in vitro shooting and rooting stage render plantlets to develop poor

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roots and shoots in terms of root hairs & cap and stomata & cuticle development respectively. The plantlets at this stage, if not dealt with expertise, become vulnerable to high percentage of damage and loss (Ortega-loezaet al., 2011). Júnior and Venturieri (2011) have reported that the germination phase in micropropagation of orchids has been fully mastered but the ex vitro phase is rarely acknowledged. They further stated that the ex vitro phase is the most difficult part of the orchids cultivation and the knowledge about the phase does exist but is kept secret (Colombo et al., 2005).

A variety of approaches such as hydroponic systems, different in vitro culture media and concentration of agar & sucrose in growth medium have been employed to reduce the loss of micropropagated orchid plantlets at acclimatization stage (Deb and Imchen, 2010). Beside these all approaches, the proper selection of soil substrates, correct shade management, irrigation and gradual lowering of humidity can solve the problems and reduce the losses during acclimatization (Deb and Imchen, 2010). The in vitro plantlets should be large and vital enough when transferring ex vitro onto an appropriate substrate and under plausible relative humidity (Lesar et al., 2012).

In the current study, an improved protocol using cheap and simple substances found in nature for acclimatization of in vitro grown orchid plantlets has been discussed.

MATERIAL AND METHOD

This study was carried out at the Biotechnology Lab., Date Palm Res. Inst., Botany Dept., Shah Abdul Latif Univ., Khairpur, Sindh, Pakistan during the period from2012 to 2013.

Substrate used

The protocol was divided into two phases: acclimatization and post-acclimatization (after 5 months of transplanting). During the first phase, 9 different substrate mixtures were tested to serve as soil beds of the in vitro plantlets under ex vitro conditions of the net house (Table 1, Figure 1). Thereafter, the soil mixture used for the second phase was categorized into a control and a treatment. The control (T^3) mixture of second phase consisted of coco-peat, hill sand and perlite at 1.0:1.0:0.2 (v/v/v), whereas the treatment (T^{10}) composed of two layers: the lower layer occupied 30% of the plastic bag volume having coco-peat, hill sand and perlite at 1.0:1.0:0.2 while the upper layer filled with wood and bark chips of different plants. It is worth to mention that hill sand is used to acclimatize Banana (Abul-Soadet al., 2012). This sand of hills found on the boundaries of 'Khairpur' district, Pakistan. It is always mixed with small stones and has to be sieved to separate the small particles looking like sand. Its color is dull yellowish brown. The pH is 5.57 and Electric Conductivity (EC) is 1.03 (dS m^{-1}).

All the substrates, except commercial coco-peat that was already sterilized, were treated by fungicide solution (Mancozeb + Metalaxyl (M/S Zhejiang Minngyun Industries Co., Ltd. Wenzhu China) before making mixtures. The soil mixtures were poured into small plastic pots of 15 cm long × 12 cm wide during the first phase and transferred into plastic bags 25 cm long × 15 wide during the second phase of acclimatization.

Plant material

The plantlets with uniform growth, 2-4 leaves and 2-3 roots from the in vitro rooting medium involved NAA at 3.0 mg l^{-1} were transferred to the net house to conduct the first trail. After then, the survived plants in acclimatization were randomly selected after five months of transplantation and shifted into larger plastic bags for post-acclimatization trail.

The acclimatization protocol

The experiments of acclimatization and post-acclimatization phase were carried out under net house conditions and the following protocol was followed:

The in vitro developed plantlets were rinsed with sterilized water to remove any media adhered to their roots.

To avoid any contamination, the plantlets were subsequently dipped into the fungicide solution at 2 g/l for 5 min. before implanted into the pots.

Immediately after potting, the plantlets were shifted under polyvinyl plastic sheet used as a low tunnel inside the net house to preserve the moisture and avoid drying.

The plantlets remained completely covered and undisturbed during the first week of the transplantation to maintain maximum percentage of relative humidity around the plantlets.

During the second week, the cover was slightly opened for 2 to 3 minutes every day to reduce relative humidity inside the tunnel.

During the third week, the cover used to be kept open for 5 minutes daily. Subsequently, the plantlets were completely uncovered at the end of fourth week of the transplantation.

The plantlets were misted twice a week during winter and twice to thrice a day during summer. The plants were watered regularly as per need according to the moistness of the substrates.

Recorded measures and statistical analysis of data

The number of surviving and dead plantlets was recorded after every 4 weeks from transplantation. The first and

Table 1. The survival percentage of the ex vitro Orchids (*Dendrobium nobile*) transplanted on different soil mixtures after 120 days.

Treatments	Coco Peat	Hill sand	Coal pieces	Date Palm chips	Brick chips	Saw dust	Perlite	Survival%
T^1	2	1	-	-	-	-	0.2	80
T^2	1	2	-	-	-	-	0.2	60
T^3	1	1	-	-	-	-	0.2	100
T^4	1	-	2	-	-	-	-	0
T^5	1	1	1	1	-	-	-	20
T^6	1	-	1	1	-	-	-	0
T^7	-	-	1	1	1	-	-	0
T^8	1	-	-	-	-	2	-	60
T^9	-	1	-	-	-	2	-	40

T1-9 stands for 1-9 different soil mixtures tested. Soil mixtures were prepared by v/v ratio.
"-" stands for "Nil".

Figure 1. Substrates used in soil mixtures during acclimatization phase; a. hill sand; b. brick chips; c. saw dust; d.date palm chips; e. coal pieces.

the second data during acclimatization stage were recorded after 30 and 70 days of transplanting (DAT) respectively. While during post-acclimatization, these were recorded after 07 and 40 DAT respectively.

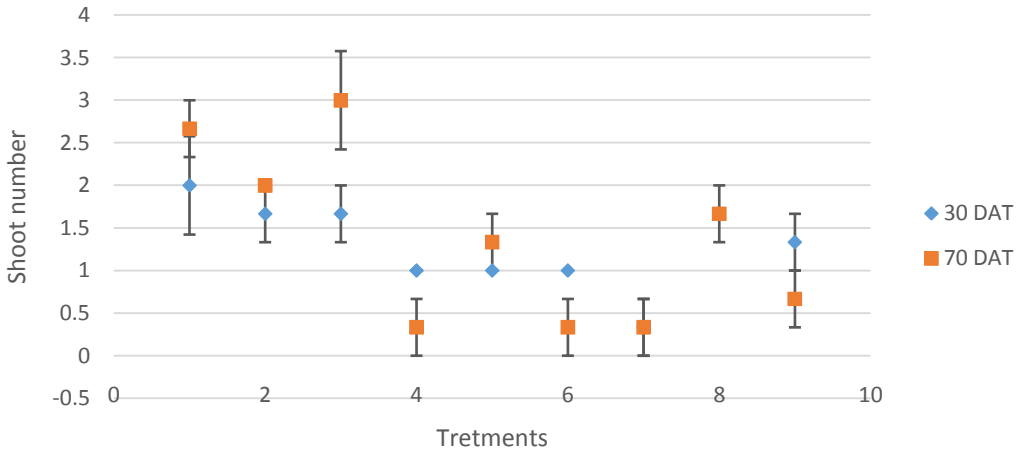
Each treatment was consisted of 6 replicates and each replicate (represented by a plastic pot/bag) contained 1 plantlet. The documented data was analyzed through ANOVA and the significance among treatment means was compared by using Standard Error & Duncan's multiple range (DMR) test at $P \leq 0.05$ (Steel et al., 1997).

RESULTS AND DISCUSSION

During acclimatization phase

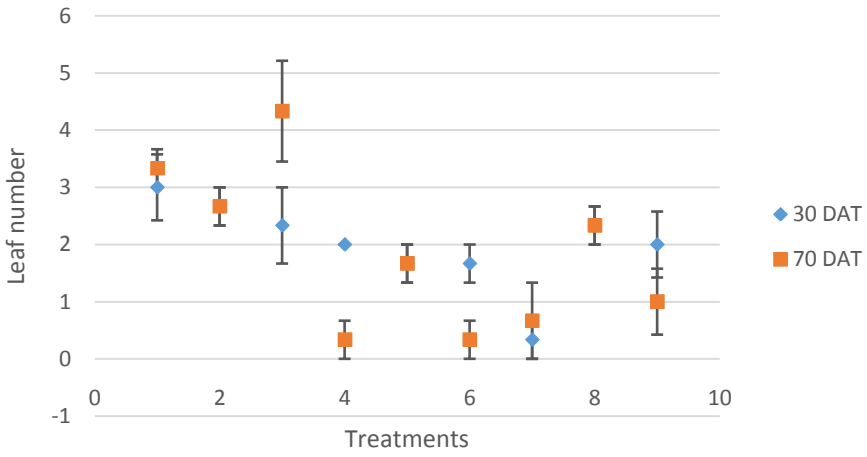
Micropropagation technique without a successful acclimatization is of no use even if it is most effective for in vitro shoot and root development. Deb and Imchen (2010) have reported that the broader use of micropropagation is mostly limited by high percentage of mortality or damage to plantlets during acclimatization. In

Figure 2. Effect of different soil mixtures on number of shoots during acclimatization.



Error bar represents standard errors.

Figure 3. Effect of different soil mixtures on number of leaf during acclimatization.



DAT stands for day after transplanting

the current study concerns the use of proper soil substrate and methodology to increase the percentage of survival and sustain healthy growth for the growing plants.

The maintenance of maximum humidity and optimum temperature around the plantlets during initial days of transplantation is of utmost importance. During in vitro cultures, plantlets are grown under high humid conditions inside jars. Whereas in greenhouse or in filed, it is much lesser than the laboratory conditions (in vitro). Therefore, a rapid change in environmental conditions, especially in terms of temperature, humidity, CO_2 concentration and irradiance, produces wilting and increases plantlets mortality rate (Pospíšilová et al., 1999).

In the present study, a maximum relative humidity of about 90-100% was maintained during the first week of

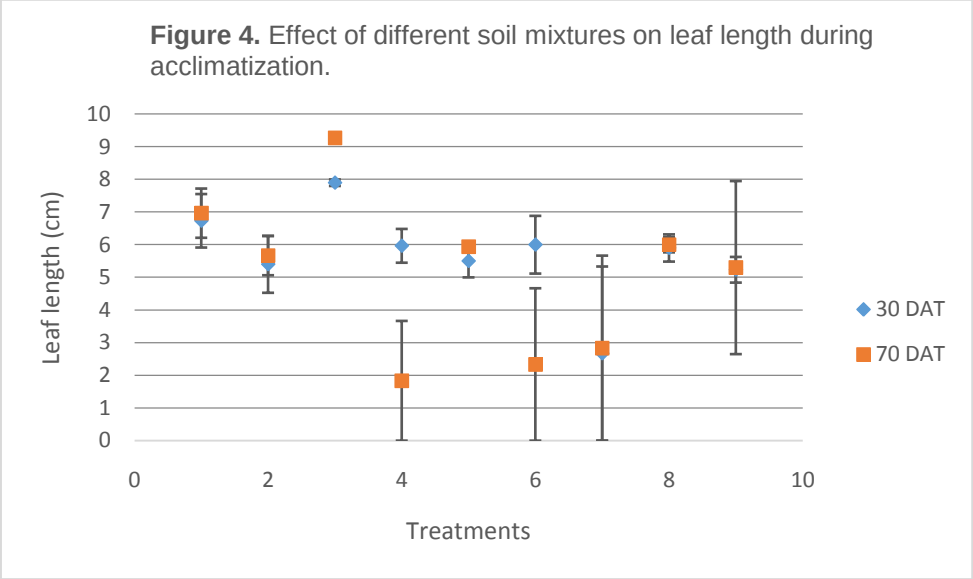


Figure 4. Effect of different soil mixtures on leaf length during acclimatization.

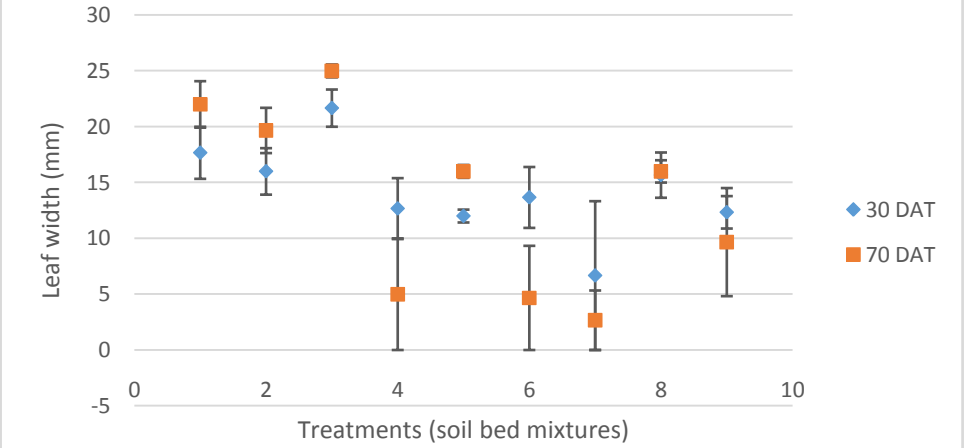


Figure 5. Effect of different soil mixtures on leaf width during acclimatization.

transplantation by keeping the transparent tunnel closed. However, for a long time, the closing of tunnel increased temperature and maximized the relative humidity, which not only damaged the plantlets but also made them vulnerable to microbial contamination. Abul-Soad (2011) reported the same problem in date palm acclimatization, and suggested decrease relative humidity gradually after 3-7 days in order to reduce fungal infection and increase the adaptability of the plants to the ambient conditions. In the current study, the tunnel was regularly opened with increasing time duration after one week of transplantation to gradually lower down the relative humidity and temperature. It was observed that the plantlets under such tunnels remained healthy and free of contamination, wilting and any type of chlorosis.

It is reported that in order to establish a successful ex vitro transplantation, the plantlets of *Phalaenopsis* and *Cattleya* must achieve a growth stage with an appropriate sprout number, foliage area, root number and root length while they are in test tubes. Beside this, the selection of a proper soil substrate with low septicity, high aeration, permeability and acidity is the key condition for initiation

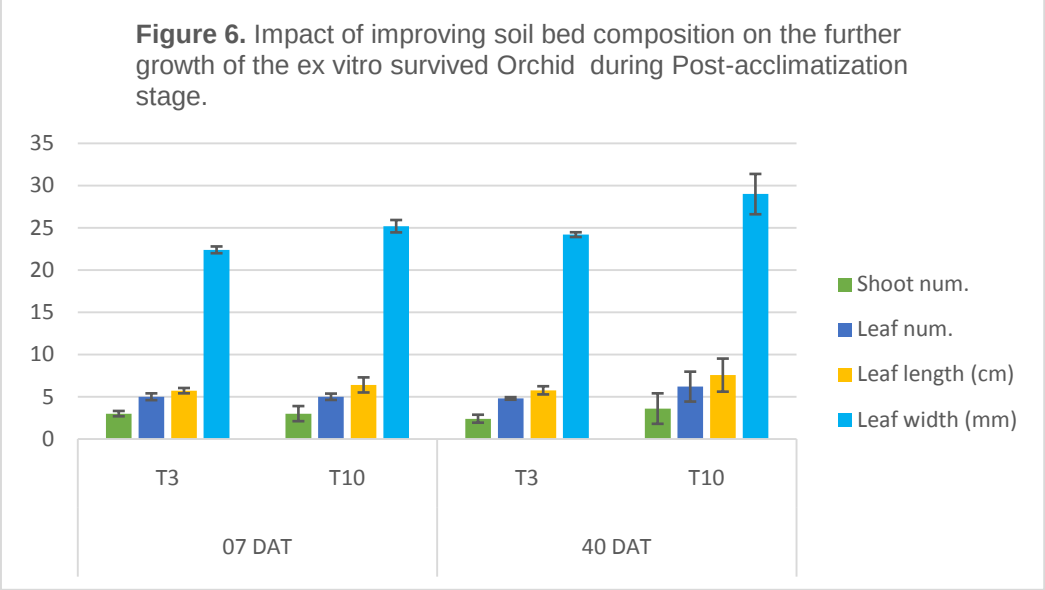


Figure 6. Impact of improving soil bed composition on the further growth of the ex vitro survived Orchid during Post-acclimatization stage.

T^3 stands for control.
T^{10} sands for treatment.

of ex vitro growth (Diaz, 2010). The soil substrate beside the medium of mechanical support to plantlets also acts as storage pool for nutrients, air and water to roots.

The data presented in figure 2-5 showed that there was a significant difference among all the treatments (soil mixtures) in respect to shoots number/plant, leaves number/plant, leaf length and leaf width. The plantlets grown in T^3 (coco-peat, hill sand and perlite; 1.0:1.0:0.2) produced 100% survivability, noted after 120 DAT (Table 1). Therefore, the survived plants on this soil mixture (T^3) were healthy and no any plant in all the replicates showed any type of leaf chlorosis or leaf fall (Figure 7a). However, in T^1, T^2 and T^8, the survival rate was 80%, 60% and 60%respectively, noted after 120 DAT. While in rest of the treatments survival rate was very low, which decreased up to 0% in T^4, $T^6$and T^7after 120 DAT. These results indicated the crucial impact of the soil mixture on successful transplantation process.

The number of shoots, leaves, leaf length and leaf width of the transplanted plants on T^3 and T^1 were significantly better than other treatments (Figure 2-5). Nevertheless, the growth rate in T^2 was not significant compared to T^3 and T^1 but it was relatively better than all other remaining treatments. The treatment number 8 (T^8) although revealed very poor growth rate, its survival rate was comparatively better than the rest of the treatments except T^3, T^1 and T^2. It was also observed that fungal contamination appeared in T^8 and T^9 only, which was perhaps due to high level of nutrient contents and unhygienic conditions in wood dust. The results in T^8 indicated that the soil substrates having high water holding capacity increased survivability of in vitro grown plantlets in ex vitro net house conditions during acclimatization. However, there was a slow growth rate due to the fungal contamination.

The results showed that for acclimatization of in vitro grown orchids, the soil substrates having high water holding capacity and moderate aeration were the best. This explains why the treatment number 3 (T^3) showed better performance among the rest of the treatments as it contained coco-peat, hill sand and perlite. The coco-peat is mostly used as a substitute of peat-moss and can absorb water up to 20% of its weight, which is suitable to improve water holding capacity of the substrate and nutrient contents at the initial stage. The hill sand is having relatively big size soil particles (Abul-Soadet al., 2012) which made good aeration and mechanical support to plantlets (Figure 1a). Whereas, the perlite is ground inert volcanic lava, which is expended at 800ºC, is also useful to provide aeration and permeability in any substrate mixture.

During post-acclimatization phase

Although in the previous stage of acclimatization stated above, there was a successful establishment of plantlets in terms of survival rate but the rate of shoot and root development was very poor and insignificant. The intention to establish this stage was to investigate the rate of vegetative growth of in vitro acclimatized plantlets on

Figure 7.(a-c). Different stages of growth during acclimatization in the net house showing new shoots and leaf formation of orchid plantlets; a. acclimatization (T^3, 120 DAT); b. post-acclimatization (T^{10}, 07 DAT); and c. post-acclimatization (T^{10}, 40 DAT).

two different soil substrates ($T^3$and T^{10}) and in order to ensure the subsequent development of the acclimatized plants.

The results presented in figure (6) revealed that there was a significant difference between treatment (T^{10}) and control (T^3). The increase in growth rate of leaf length and width was significantly better in T^{10} as compare to T^3 (Figure 7c). Moreover, the formation of new shoots, leaves and roots was also only observed in T^{10} (Figure 8, left). It was observed that the in vitro developed leaves and roots had stopped growing further and new leaves and roots replaced them, which were very healthy and vigorous. Pospíšilová et al., (1999) reported similar results that in most of the plant species leaves formed in vitro do not grow further but are replaced by new leaves in ex vitro conditions.

On the other hand, it was observed that the high moisture contents and less aeration to roots for long time in T^3 delayed their development and new root formation. Moreover, the in vitro formed roots were being damaged in T^3 as showed drying out started from tips to base (Figure 8, right).

In general, the growth was very slow in terms of new shoot or leaf and root formation on T^3 during post-acclimatization stage. Results in current study showed that during post-acclimatization stage, there was very rapid rate of new root and shoot formation produced on T^{10} compared to T^3. It shows that the transfer of plantlets to more aerated substrate (T^{10}) after their adaptation to ex vitro conditions (acclimatization) proved very significant for their subsequent rapid shoot formation and root development. These results were confirmed by Pospíšilováet al., (1999) and Van Huylenbroeck and De Riek (1995) who stated that during acclimatization of *Spathiphyllum floribundum* two different stages were observed; an adaptation period with slow shoot growth & root formation, followed by a period of fast growth of roots and shoots.

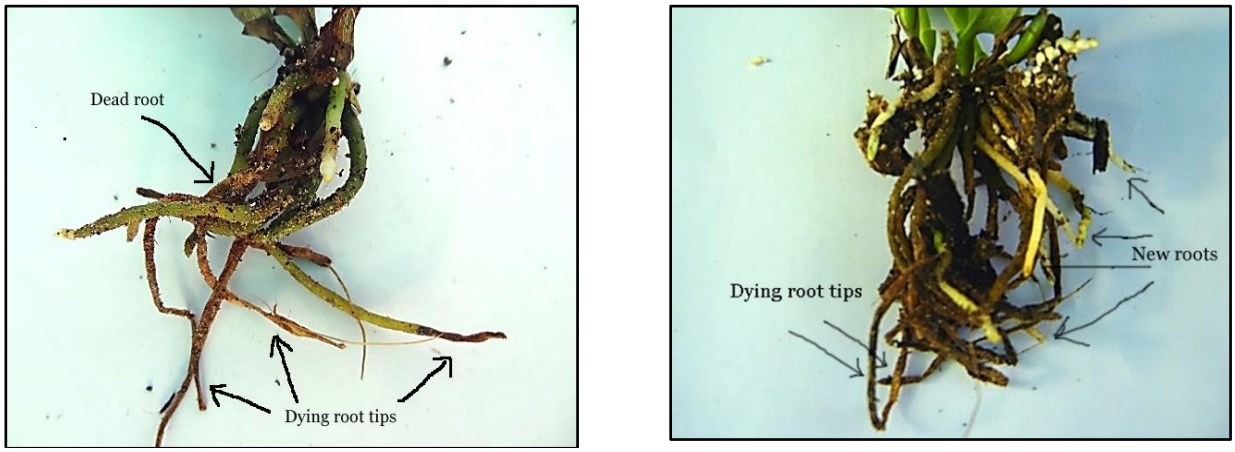


Figure 8. Formation of new roots on T10 (left),and drying out the roots onT3 (right) during post-acclimatization under net house conditions.

CONCLUSION

In the current study a protocol was developed for an economic and efficient acclimatization of in vitro grown plantlets to ensure subsequent growth of Orchid. The achieved results revealed that most of the ex vitro plants showed two stages of growth after successful transplantation: a stage of adaptation to ex vitro conditions associated with slow growth followed by a stage of rapid growth during their acclimatization. During the first phase a need of high humidity around the transplanted plantlets for few days which should be decreased gradually to normal ambient conditions. The best soil substrate having high water holding capacity, moderate porosity or aeration and low contamination rate composed of coco-peat: desert sand: perlite at ratio 1.0:1.0:0.2 (v/v/v). In the second phase (post-acclimatization phase), rapid growth occurred with a necessity to change the soil bed composition into low water holding capacity and higher porosity or aeration. The current protocol can be applied for orchids as well as for other similar plant species.

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