Original scientific paper
10.7251/AGRENG1803005K
UDC 582.594

INVESTIGATING THE EFFECT OF SUBSTRATE, MYCORRHIZAL APPLICATION AND BULB SEPARATION ON THE GROWTH OF THE WILD ORCHID *ANACAMPTIS PYRAMIDALIS*

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ABSTRACT

Pyramidal orchid (*Anacamptis pyramidalis*) is a wild terrestrial orchid widely found in Lebanon and the Mediterranean zone. Random collection and trade of the orchid for medicinal and edible use (salep) subjected it to a risk of extinction. Consequently, the current work aimed to propagate this orchid in vivo. Orchid bulbs were transplanted from the wild into four different soil substrates (Pinebark, Pinebark-Peat (1/1), Peat-Sand (1/1) and control: soil from the collection site) under controlled conditions of temperature and humidity. The effect of mycorrhizal application (MY: Yes) was tested in the different substrates except in control and orchid bulbs (son bulb and mother bulb) were planted combined (SB+MB) or after separation of the mother bulb (SB). Same treatments were repeated over two consecutive years. Results showed an earlier emergence of son bulbs (SB) grown in control substrate. Moreover, there was a significant difference in plant growth with superiority for (SB) compared to (SB+MB) regarding plant length and elongation of first leaf except bulb dimensions (length and width) that were higher in (SB+MB). Mycorrhizal application enhanced the overall growth of plants and its effect was the most obvious in the substrate Peat-Sand (1/1). In general, the best growth of the orchid was observed at the level (SB)/(MY:Yes)/Peat-Sand (1/1). Results of the second experimental year confirmed those of the first year with an improvement of the rate of emergence by 13%. The in vivo propagation method was beneficial on improving the growth of A. *pyramidalis* ex situ and it could be adopted as an initiative for wild orchid conservation.

Keywords: *Anacamptis pyramidalis, conservation, substrate, mycorrhiza, bulbs.*

INTRODUCTION

The Mediterranean region is one of the richest zones by its fauna and flora. Lebanon a part of this zone presents one of the best examples where a landscape and floristic diversity is found due to its geographical location and high variability of abiotic conditions (Jomaa, 2008). Wild orchids are among this richness, where more than 87 species are found (Bou Dagher Kharrat, 2010), among which several species are considered as endemic in the east Mediterranean zone. The genus *Anacamptis* (Orchidaceae) consists of 11 species (Kretzschmar *et al*., 2007) and was first established by the French botanist Louis Claude Marie Richard (1754-1821) in 1817, based on *Anacamptis pyramidalis* (L.) Rich., the well-known Pyramidal Orchid (Wood and Ramsay, 2004). Pyramidal orchid is characterized by an erect stem, linear basal leaves, purple pink flowers in conical spike and ellipsoidal bulbs (Tohme and Tohme, 2014). In general, the Mediterranean terrestrial orchids including Pyramidal orchid have consistent annual growth pattern, beginning and ending by one or more dormant bulbs over summer during the drought period. Bulbs re-sprout in the following autumn and leaf formation begins in early winter and continues during winter cold season (Brundrett, 2014). This species is threatened under large scale collection pressure due to its economic value which requires the reproduction trials inevitably (Sevgi *et al*., 2012). It is strictly protected in some European countries such as Czech Republic and Slovak Republic where it is under the risk of extinction. It is simultaneously protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Štajner *et al*., 2010). In Lebanon, *Anacamptis pyramidalis* is found in different regions like Quamouaa, Ehden, Boutmeh, Kfarhouna and others (Tohme and Tohme, 2014). Although this orchid is widely found in Lebanon, it is under extinction risk because of habitat alteration and random collection for edible or medicinal use.

Therefore, the main objective of this study was to provide a propagation tool for the pyramidal orchid by transplanting it from the wild in order to be grown under specific experimental conditions and to explore its behavior after it had been subjected to different growing techniques; like the type of substrate, mycorrhizal application and separation of its bulbs. Consequently, to study the effect of the different growing techniques and their interactions on the growth of the plant.

MATERIALS AND METHODS

Orchid collection

The behavior and growth habit of the wild orchid were assessed over 2 experimental years, 2014 and 2015. Pyramidal orchids were collected from the region of Wedeh el Karem-Mount Lebanon/Lebanon, situated at an altitude of 1140 m (33° 57’ 0" North, 35° 45’ 0" East), where they were found in a high density. In each experimental year, 140 plants were collected in early June during the flowering stage of the studied species (Lind *et al*., 2007). Entire plants were collected consisting of underground parts: a root system and two bulbs (a mother bulb (MB) that has already sprouted and given the inflorescence of the current season and a new bulb that will sprout in the next season after a dormancy period that was used in this study and referred to as son bulb (SB)) as well as aboveground parts (stem, leaves and flower). However, only bulbs were used as planting material.

Experimental design and treatments

The experimental design (Figure 2) included 14 treatments with 10 bulbs per treatment. Son bulbs were planted with or without the mother bulb ((SB) or (SB + MB)) in four types of substrates (Own soil, Pine, Pine+Peat and Peat+Sand) with or without mycorrhizal application (MY: Yes or MY: No). Own soil substrate represented the soil collected at a depth of 30 cm from the site where orchids were found. This substrate was considered as "Control". Pine substrate was formed by pieces of pine bark collected from the same site and cut into small pieces prior to use. Pine+Peat and Peat+Sand substrates were prepared by mixing peat with pine bark pieces and sand respectively in a ratio 1:1 in terms of volume. Substrates properties are represented in the following Table 1.

Table 1. Composition of different tested substrates

	Own Soil	Pine	Pine+Peat (1/1)	Peat+Sand (1/1)
Ph	7.57	5.96	5.77	7.51
EC ($mS.cm^{-1}$)	0.832	0.884	0.574	0.174
Organic matter (%)	4.6	76.3	84.3	7.3
Nitrogen (kjeldahl)(%)	0.6	0.6	0.67	0.326
P_2O_5 total (digestion) (ppm)	13.71	2771.77	424.61	67.09
K_2O total (digestion) (ppm)	375	1066.35	1531.46	588.61
CaO total (digestion) (%)	9.4	6.1	4.3	1.9
MgO total (digestion) (%)	1.4	0.7	1	0.3

The application of commercial mycorrhiza took place after bulb plantation in pots of 15 cm in diameter for all pots in various substrates except in the Own Soil substrate where mycorrhizal symbiosis was found naturally. Pots were put in a climate chamber where a constant temperature was maintained at 5°C during November, December and January, and then it was raised to 10^{o} C in February and to 15°C during March and April. Temperatures were fixed in a way to provide the optimal natural values during the growth of the pyramidal orchid in the wild. Substrates were continuously wetted by spraying water in order to preserve a high humidity level in the growing medium of bulbs.

Data recording

The experiment in both years was carried out over 6 months; from emergence to the end of the vegetative growth prior to flowering. Several parameters were recorded in order to evaluate and compare the emergence and growth of plants. The date of emergence was the number of days between transplantation date in first of July and the date of appearance of the first shoot tip at the soil level. The rate of emergence revealed the percentage of emerged bulbs among the total planted bulbs. The elongation of longest leaf was assessed by 3 readings (1, 2 and 3 months after emergence), and the stem length was assessed twice during the growing cycle (respectively 4 and 5 months after the emergence date). The timing of each reading was adopted in order to cover a part of the life cycle of *Anacamptis pyramidalis*, starting by bulb emergence followed by leaf formation and ending with stem elongation. The experiment was stopped at this level in order to assess the bulb growth variation as affected by different factors. The bulb normally reaches its optimal growth prior to flowering. Therefore, bulb dimensions (length and width) were measured at the end of the experiment after plant removal from pots.

Statistical analysis

The effects of different factors (substrate types, mother bulb separation v.s. no separation and mycchorizal addition v.s. mycchorizal absence) and their interactions on the averages of the measurements were analyzed using Factorial ANOVA. For the elongation of the longest leaf and the plant length Repeated Measures Factorial ANOVA were applied to also study the time (readings) effect.

RESULTS AND DISCUSSION

Rate of emergence

The planted bulbs started to sprout and emerge above soil level after several months of dormancy. Some bulbs did not sprout. The emergence rates were 69 % and 82 % in the first and second experimental years respectively.

Date of emergence

Results showed a variation in the emergence period of potted bulbs (Figure 1). In the control substrate (Own Soil) an earlier bulb emergence was observed compared to other substrates. In this substrate and for both experimental years, the average date of emergence was higher in (SB+MB) than in (SB) (134 days and 130 days respectively). In general, for all substrates the mother bulb separation prior to plantation has led to an earlier emergence. Averages obtained in (SB) were lower than those in (SB+MB) in all substrates in case of no mycorrhizal application (MY:No) (135, 137 and 133 days in (SB) against 138, 142 and 139 days in (SB+MB) for the substrates (Pine), (Pine+Peat) and (Peat+Sand) respectively). Mycorrhizal application affected the date of emergence only in the substrate (Pine+Peat) in (SB) inducing an earlier emergence with a difference of 6 days observed between MY:Yes (131 days) and MY:No (137 days).

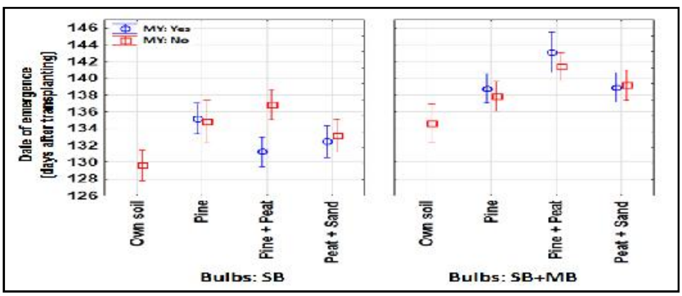

Figure 1. Variation of the average emergence under the effect of substrates type, mother bulb separation and mycorrhizal application in both years.

Elongation of longest leaf

This parameter was affected by the different investigated factors (Figure 2). The average elongation of longest leaf varied between substrates. For instance, in the second year experiment it was significantly the highest in the substrate (Own Soil) for (MY:No) in the final reading in February for (SB) and (SB+MB) with 23 cm and 22 cm respectively. There was a positive effect of mycorrhizal application on the elongation of longest leaf, in specific in the first year experiment for the substrate (Peat+Sand) with separation of mother bulb (SB), where an average of 20 cm was reached in (MY:Yes) compared to 15 cm in (MY:No). In addition, results showed that planting the son bulb alone (SB) has improved this parameter despite the mycorrhizal application. This was evident in the second year experiment, regarding the substrate (Peat+Sand) where a final average of longest leaf elongation of 16 cm was reached in (SB) compared to 8 cm in (SB+MB) although mycchoriza was absent (MY:No). Moreover, the combination of mother bulb separation (SB), mycchorizal application (MY:Yes) and (Own Soil) provided the best results with regards to this parameter; the highest average (24 cm) was recorded at the level (SB)/(MY:Yes)/(Own Soil) in the second experimental year.

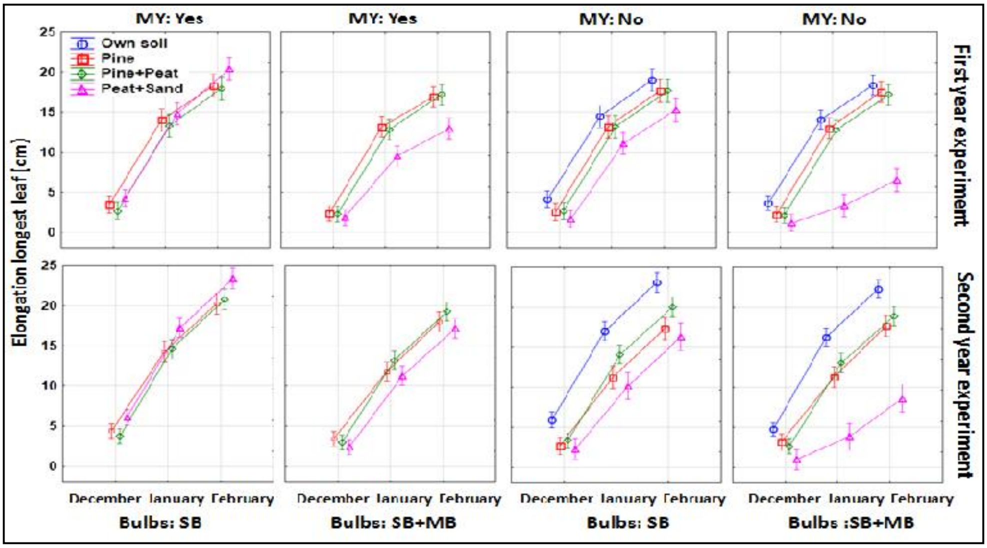

Figure 2. Averages (markers) and the 95% limits of confidence (vertical bars) of the elongation of longest leaf for the different levels of the experimental factors.

Plant length

The effects of mycorrhizal application and mother bulb separation showed an increase in plant length especially for this parameter especially when added to the effect of tested substrates (Figure 3). In case where bulbs were cultured without any additional treatment; no mother bulb separation (SB+MB) or no mycorrhizal application (MY:No), the control substrate (Own Soil) performed the best when compared to the other three substrates with a significant difference in the first experimental year. The substrate (Peat+Sand) showed the lowest average for both experimental years; for (MY:No)/(SB+MB) the final average of plant length scored 14 cm and 20 cm respectively in the first and second experimental years. The mycorrhizal application gave effective results when tested alone without considering the effect of mother bulb separation. Its effect was the most obvious in the substrate (Peat+Sand) especially in the second year experiment where an increase of 20 cm was observed after mycorrhizal application for the level ((SB+MB)/(Peat+Sand)/(My:Yes)) where plants scored an average length of 40 cm compared to 20 cm for the same level without mycorrhizal application ((SB+MB)/(Peat+Sand)/(MY: No)). In addition, a positive effect of mother bulb separation was observed when tested alone without mycorrhizal application (MY:No). In the first year, in the substrate (Pine+Peat), the final average scored 34 cm in case of mother bulb separation while the average decreased to 29 cm in case of no mother bulb separation in the same substrate. Finally, plant length was influenced the most by the combination of mother bulb separation and mycorrhizal application in the substrate (Peat+Sand). For instance, in the second experimental year, the highest plant length was recorded at the level (SB)/(MY: Yes)/(Peat+Sand) with an average of 44 cm.

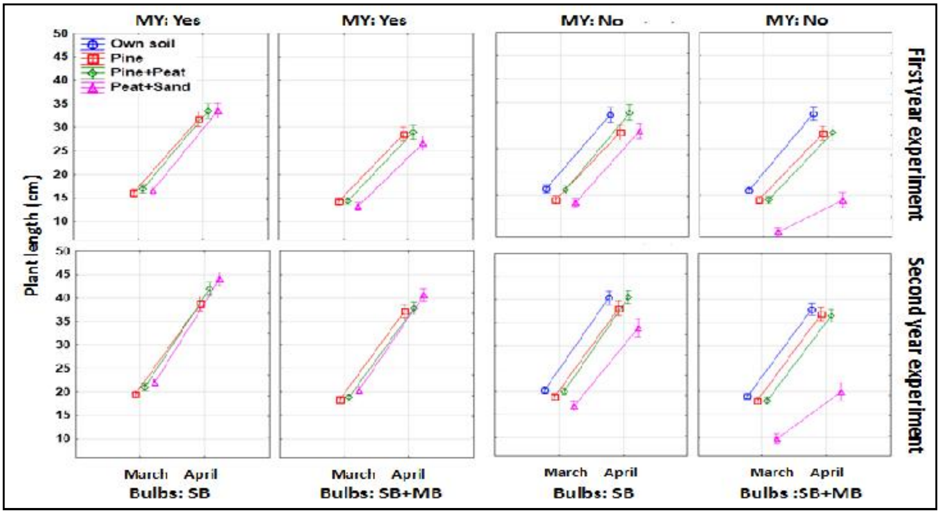

Figure 3. Averages (markers) and the 95% limits of confidence (vertical bars) of the plant length for the different levels of the experimental factors.

Bulb dimensions

It was found that bulb length and width were significantly higher in the second year experiment compared to the first year despite the effects of substrates, mother bulb separation and mycorrhizal application (Figure. 4). Mycorrhizal application enhanced bulb length and width and its effect appeared in all substrates despite the presence or absence of the mother bulb. In specific, it increased bulb length by 1.2 cm and bulb width by 0.3 cm in the level (SB+MB)/(MY:Yes)/(Peat+Sand) compared to (SB+MB)/(MY:No)/(Peat+Sand) in the second experimental year. On the other hand, the effect of mother bulb separation on bulb dimensions differed according to the substrate type. On the other hand, the effect of mother bulb separation on bulb dimensions differed according to the substrate type. For instance, in the second year experiment the mother bulb separation affected negatively bulb dimensions in the control Own Soil substrate; the bulb length and width scored in the level (SB+MB)/MY:No/Own Soil an average of 3.4 cm and 1.3 cm respectively, while this average decreased after mother bulb separation in the level (SB)/MY:No/Own Soil and scored an average bulb length and width of 3cm and 0.9 cm respectively. For the Pine substrate the mother bulb separation did not have any significant effect especially for bulb length; in the second year experiment, the same average bulb length was obtained (2.6 cm) for both levels: (SB+MB)/MY:No/Pine and (SB)/MY:No/Pine.

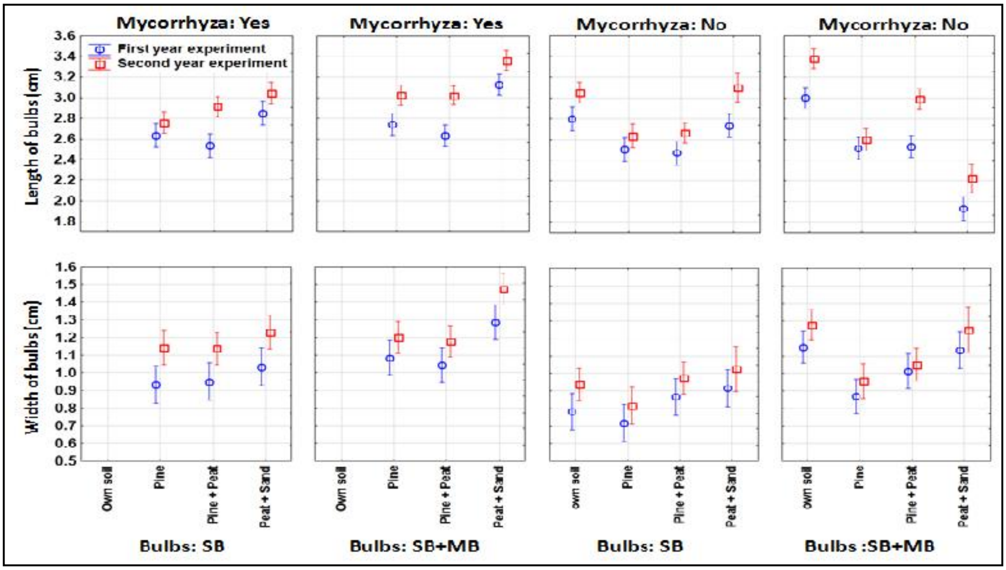

Figure 4. Averages (markers) and the 95% limits of confidence (vertical bars) of the bulb dimensions for the different levels of the experimental factors.

Anacamptis pyramidalis was successfully transplanted from the wild and grown under experimental conditions. The rate of emergence was improved by 10% when the mother bulb was kept and an earlier emergence of 3 to 8 days was observed when it was removed. This earliness observed for (SB) compared to (SB+MB) could be attributed to bulb maturity and its dormancy stage at the time of plantation. It seemed that the separation of the mother bulb have stimulated an earlier breakage of dormancy in the son bulb which might have caused an earlier emergence. On the other hand, planting bulbs in (Own Soil) while controlling temperature and humidity provided conditions of growth that were similar to the natural environment of bulbs in the wild. Microorganisms present naturally in (Own Soil) were absent in the other tested substrates which might have induced an earlier date of emergence. Moreover, the positive effect of mother bulb separation and mycorrhizal application was obvious for the elongation of longest leaf and plant length in both experimental years regarding all substrate types except for the control (Own Soil) where mycorrhizal application was not adopted and where the mother bulb separation did not affect significantly the average stem length in both years. This could be related to the natural behavior of the Pyramidal orchid in its wild ecosystem. *Anacamptis pyramidalis* in the wild could reach a high length with one or even 2 bulbs. According to Sevgi *et al*. (2012) a length of 65.5 cm could be reached. The combination of mother bulb separation and mycorrhizal application enhanced the growth especially in the substrate (Peat+Sand) which was poorer in inorganic nutrients (N,P,K) compared to (Pine) and (Pine+Peat) substrates provided due to the fact that the beneficial effect of mycorrhiza is often associated with the low availability of inorganic nutrients (Dhillion and Friese, 1994). On the contrary, in case of mycorrhizal absence the high availability of organic matter and nutrients in the substrates (Pine) and (Pine+Peat) allowed plants to reach higher heights. Using (Own Soil) also appeared to enhance the elongation of the longest leaf compared with other substrates although they were richer in organic matter. This could be due to its higher pH (7.57) compared with those of (Pine) (pH=5.96) and (Pine+Peat) (pH=5.77) substrates. In fact, *Anacamptis pyramidalis* is found and prefers alkaline soil (Heinsoo, 2012), with a pH of 7.01 (Tsifsfis *et al*., 2008). Concerning bulb dimensions, mycorrhizal application enhanced the bulb growth while mother bulb separation had a variable effect depending on the substrate type. In addition, the (Own Soil) (Control) representing the natural substrate of Pyramidal orchid induced the best growth due to the adaptation of the bulb to this type of soil in nature. Finally, the mortality that happened on a relatively small percentage of bulbs could have been caused by some pathogenic factors in bulbs that could not be detected prior to plantation or to the normal difficulties that face the transplantation of a wild plant from the nature to be grown under experimental conditions.

CONCLUSIONS

Conclusions from this study were:

-Transplantation through different vegetative parts such as rhizomes, bulbs, and stolons is the simplest method for wildlife conservation compared to other conservation methods (cryoconservation, in vitro). Its adoption is recommended at local and national levels seeking to protect and conserve various wild species that could be threatened in degraded ecosystems or under risk of extinction.

-The success of in vivo experiments could provide a tool not only for conservation purpose but also for mass scale production.

-Re-introduction trials in the wild of ex situ propagated orchid plants could be investigated in future research studies.

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