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In vitro seed germination, plantlet growth, tuberization, and synthetic seed production of *Serapias vomeracea* (Burm.f.) Briq.

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Abstract: Orchids are considered recalcitrant plants in in vitro propagation. Due to the lack of appropriate micropropagation techniques for mass production and damage to their ecological distribution posed by local gatherers, these species are threatened with extinction, including *Serapias vomeracea* (Burm.f.) Briq. In this research, we put forward a complete micropropagation method covering in vitro micropropagation, synthetic seed formation, germination in soil, and acclimatization to ambient conditions. To the best of our knowledge this is the first report of successful synthetic seed formation and germination of *S. vomeracea*. Initially, seeds were germinated in different culture media and also media supplemented with different concentrations of plant growth regulators. Effects of plant growth regulators on tuber formation, glucomannan contents, and different growth parameters were evaluated throughout the study. The best germination rate (84.03%) was achieved on Orchimax including activated charcoal medium and supplemented with 2.0 mg/L 6-benzyladenine. The longest shoot elongation amongst plantlets was observed on the same medium supplemented with 0.25 mg/L thidiazuron, whereas 2.0 mg/L indole-3-butyric acid favored leaf formation. Higher indole-3-butyric acid concentrations were found to be more effective in the formation and elongation of roots. Orchimax medium supplemented with zeatin (2.0 mg/L) was superior to the others in terms of tuber formation and glucomannan content therein. Adaptation of seedlings to soil conditions and germination abilities of synthetic seeds were also studied and seedlings were successfully acclimatized and adapted to soil conditions.

Key words: *Serapias vomeracea*, micropropagation, synthetic seed

1. Introduction

The Orchidaceae are one of the most widely distributed families of flowering plants in terms of the number of various families on earth with approximately 30,000 species in 800 genera (Arditti and Ghani, 2000). The first thing that comes to mind about orchids is that they are expensive ornamental plants with flowers in various shapes and colors native to tropical regions. There is also an orchid group named salep plants growing naturally in some countries, including Turkey, the tubers or corms of which are utilized to make hot beverages and ice cream.

Germination and plantlet formation from salep seeds is extremely difficult and takes a long time in their natural environment. They have the smallest seeds among all flowering plants (Mitchell, 1989). They are covered with a thin seed coat consisting of a transparent and loose layer and these seeds either lack or possess a minute amount of endosperm tissue. Therefore, for germination they need to establish a mutual interaction with mycorrhizal fungi in addition to the requirement of gentle environmental conditions concerning heat, light, moisture, and oxygen in order to germinate. In addition, mass production opportunities with applied vegetative methods are either limited or do not exist at all. As a result, cultivation of salep orchids, such as *Serapias* species, is dramatically restricted.

The below-ground vegetative units are the tubers, which are usually found in couples (sometimes 3–6 parts) that are attached to each other. One of these tubers gives rise to the current year's vegetation, while the other is actually a remnant from the previous growth season. Besides such vegetative growth, these orchid tubers are collected for salep production. As mentioned above, it is an additive in the food industry. The main ingredients of orchid tubers are glucomannan (7%–61%), starch (1%–36%), sugar (2%–3%), and nitrogenous substances (0.5%–1%). The quality of salep varies based on the type of orchid; high quality salep is obtained from orchids that contain 40% or more glucomannan (Sezik, 1984).

Uncontrolled and excessive collection of these orchid tubers, due to their economic value, and dismantling of the plant without allowing the maturation of seeds depress local populations of salep species and even drive

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some species to near extinction. Fast and controlled reproduction of these plants has become mandatory since these plants cannot be cultivated and produced vegetatively. It has been accepted all over the world that the most ideal alternative method that can be applied for the protection of plants collected from the wild for commercial purposes is production. Generating plants using tissue culture techniques (micropropagation) enables fast production of orchid species in large quantities as they are used in the production of many plant species (Yamazaki and Miyoshi, 2006; Valletta et al., 2008; Bektaş et al., 2013). If appropriate nutrient, hormone, and culture requirements of the plant are provided sufficiently, it is possible to produce all plant species using micropropagation techniques (Mansuroğlu and Gürel, 2001). For this purpose, some studies towards developing tissue culture systems of some plants of the family Orchidaceae under in vitro symbiotic and asymbiotic conditions were conducted (Yamazaki and Miyoshi, 2006; Valletta et al., 2008; Bektaş et al., 2013).

The present study aimed to develop protocols for efficient production of *Serapias vomeracea* (Burm.f.) Briq. of the family Orchidaceae by using plant tissue culture techniques such as micropropagation and synthetic seed production.

2. Materials and methods

2.1. Surface disinfection and germination of seeds

Mature *S. vomeracea* seeds growing in nature were used for the initiation of culture. These seeds were obtained from the Aegean Agricultural Research Institute (İzmir, Turkey). The surface disinfection of mature seeds was carried out by following the method described by Bektaş et al. (2013). To perform the experiments on in vitro germination, four basal media with different composition were used (Table 1). The effects of different plant growth regulators [indole-3-acetic acid (IAA), indole-3-buyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), zeatin (ZEA), 6- benzyladenine (6-BA), and thidiazuron (TDZ)] at different concentrations (0.25, 0.5, 1.0, and 2.0 mg/L) on germination were investigated. In order to compare the efficiency of plant growth regulators on germination, the culture media without any plant growth regulators (PGRs) were used as negative controls. The culture dishes were incubated at 23 ± 2 °C for 3 months in a growth chamber set to 16/8 h photoperiod and a light density of 32 µmol s^{-1} m^2. Seed germination rates (percentages) were recorded 2 months after sowing the seeds in the culture dishes. At the end of the third month, the percentages of protocorm formation were determined.

2.2. Shoot formation

The effects of various PGRs on the length of shoots, and number of leaves and roots of *S. vomeracea* seedlings grown under in vitro conditions were investigated. ZEA, KIN, 2-iP, 6-BA, TDZ, IBA, IAA, and 2.4-D were used as PGRs at different concentrations (0.25, 0.5, 1.0, and 2.0 mg/L). The protocorms formed from germinated seeds were used as explants. The lengths of shoots were determined 60 days after planting the protocorms. After 3 months of culture, root numbers and lengths and the number of leaf of the seedlings were evaluated. Each experiment was designed as 3 replicates with 20 samples.

2.3. Synthetic seed production

2.3.1. Formation of protocorm-like bodies

Protocorm like bodies were formed in the Orchimax medium including activated charcoal and supplemented with different concentrations of TDZ alone (0.1, 0.25, 0.5, 1.0, and 2.0 mg/L) or in combination with 1.0 mg/L IBA. The protocorms obtained from germinated seeds were used as explants. Protocorms were longitudinally divided into two and planted in the medium. The cultures were incubated at 24 ± 2 °C for 3 months in a growth chamber set to 16/8 h photoperiod and a light density of 32 µmol s^{-1} m^2. After 8 weeks of culture, the PLB formation rate and mean number of the PLB formation for each explant were determined.

2.3.2. Preparation of coating matrix and encapsulation of the PLB

Sodium alginate (3%) solution was prepared for encapsulation of the protocorm-like bodies and $CaCl_2$ (75 mM) was prepared for solidification of the synthetic encapsulation matrix, respectively (Aquea et al., 2008; Daud et al., 2008; Geetha et al., 2009; Sarmah et al., 2010; Nor Asmah et al., 2011). The chemical content of Orchimax medium without activated charcoal was used as the synthetic culture medium (artificial endosperm). In addition, this culture medium was supplemented with 2.0 mg/L ZEA and 1.0 mg/L IBA, respectively.

2.3.3. Germination of synthetic seeds

The ability for germination and formation of seedlings from synthetic seeds was evaluated in the culture medium (under in vitro conditions) and soil conditions. The Orchimax medium including activated charcoal (+OM) without any PGRs was used as in vitro germination medium. Commercial peat (Cihan Agriculture, Manisa, Turkey), which was sterilized, was used as soil culture medium (analysis values: pH 5.5–6.5, organic matter content: 69%, moisture: 53.43%, and water holding capacity: 618). At the end of 3 months, the viability and germination rates of synthetic seeds were estimated in all tested conditions.

2.4. Acclimatization of seedlings

The seedlings, which became ready to be transferred to the soil, were cleaned until no residue remained and were planted in the sterilized peat medium. The lids of the culture dishes were closed to eliminate the risk of

Table 1. Culture media and chemical contents

		–OM	KCM	LM	+OM
Macroelements mg/L	$CaCl_2$	166.00	-	-	166.0
	KH_2PO_4	85.00	250.00	135.00	85.00
	KNO_3	950.00	-	-	950.0
	$MgSO_4$	90.35	122.15	58.98	90.35
	NH_4NO_3	825.00	500.00	-	825.00
	$Ca(NO_3)_2$	-	241.30	347.20	-
	KCl	-	250.00	1050.00	-
	$(NH_4)_2SO_4$	-	500.00	1000.00	-
Microelements mg/L	$FeSO_4.7H_2O$	-	25.00	-	-
	$MnSO_4.H_2O$	8.45	5.68	-	8.45
	$AlCl_3.6H_2O$	-	-	0.56	-
	$CuSO_4.5H_2O$	0.0125	-	0.02	0.0125
	Fe citrate	-	-	4.40	-
	H_3BO_3	3.10	-	1.01	3.10
	KI	0.415	-	0.10	0.415
	$MnSO_4.H_2O$	8.45	-	0.05	8.45
	$NiCl_2.6H_2O$	-	-	0.03	-
	$ZnSO_4.7H_2O$	5.30	-	0.57	5.30
	$CoCl_2.6H_2O$	0.0125	-	-	0.0125
	FeNaEDTA	36.70	-	-	36.70
	$Na_2Mo_4.2H_2O$	0.125	-	-	0.125
Vitamins mg/L	Myo-inositol	100.00	-	-	100.00
	Nicotinic acid	1.00	-	-	1.00
	Pyridoxine HCl	1.00	-	-	1.00
	Thiamine HCl	10.00	-	-	10.00
	Glycine		-	-	-
Organics g/L	Sucrose	20.0	-	-	20.0
	Tryptone	2.0	-	-	2.0
	Activated charcoal	2.0	-	-	
	MES (mg/L)	1000.0	-	-	1000.0

–OM: Orchimax without activated charcoal, KCM: Knudson C orchid medium, LM: Lindemann orchid medium, +OM: Orchimax including activated charcoal

dehydration and possible contamination and the dishes incubated for 30 days. The lids were reopened after 30 days of being closed and development of the seedlings was maintained in this way. The survival rate of the seedlings was determined at the end of 90 days.

2.5. Determination of glucomannan content of tubers

The amounts of glucomannan in tubers derived from different in vitro conditions were determined by the phenol–sulfuric acid method reported by Chua et al. (2012). The tubers were powdered via traditional methods. The tubers, which were boiled for 10 min in boiling water, were dried in an oven set to 50 °C. The dried tubers were crushed into powder using a mill.

Solutions containing 0, 16, 32, 48, 64, and 80 μg/mL glucose were placed into test tubes and the final volume was completed to 2 mL. Then 1 mL of 5% phenol reagent was added to the samples in the test tubes. Next 5 mL of concentrated sulfuric acid (95%–97%) was added to the test tubes, which were then incubated for 10 min at room temperature and then kept at 25 °C for 20 min. Absorbance of the samples was measured by a spectrophotometer at 490 nm and the glucose absorbance curve corresponding

to absorbance was plotted. The mannose calibration curve was determined by the method followed for glucose. The tubes not containing glucose (0 μg glucose, 2 mL of distilled water) were used as blanks.

Next 0.05 g of powdered salep material was stirred on a magnetic stirrer in 40 mL of distilled water at room temperature for 4 h. At the end of 4 h, the final volume was completed to 50 mL and the resulting solution was centrifuged (25 °C, 4000 × *g*, 40 min). Four milliliters of solution obtained from supernatant was completed to 50 mL with distilled water. Then glucomannan content was determined by phenol–sulfuric acid method. The glucomannan content of materials obtained from each culture medium, where tuber formation was observed, and differences between these culture media were also determined.

2.6. Statistical analysis

The data were statistically analyzed using SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance was used to calculate statistical significance and the significance of difference among the means was determined using one-way ANOVA and Duncan mean separation at 0.05 probability.

3. Results and discussion

3.1. Seed germination and shoot formation

Of all the basal culture media tested, the highest germination rate was observed when mature seeds of *S. vomeracea* were placed on the +OM culture medium (73.74%) for shorter time periods (20 days) (Table 2). Germination rates in Knudson C orchid medium (KCM) and Lindemann orchid medium (LM) were similar with the lowest germination rates and longest germination time. Less than 5% of orchid seeds can germinate in their natural environments and they wait for at least favorable climatic conditions to germinate. Given this information, germination efficiency is higher with +OM culture medium in terms of germination rate and germination time. In a previous study by Bektaş et al. (2013), conducted on *Orchis coriophora* L., similar results were reported, i.e. the highest germination rate was also obtained with Orchimax including activated charcoal culture medium (+OM).

–OM and +OM culture media contain an inorganic nitrogen source in addition to an organic nitrogen source (tryptone). This may be the reason why these culture media express higher germination rates. In previous studies, the germination rates of orchid seeds were higher in culture media that contained an organic nitrogen source (Stewart and Kane, 2006). Moreover, the germination of orchid seeds is inhibited by the presence of inorganic nitrogen (Raghavan and Torrey, 1964; Van Waes and Debergh, 1986; Malmgren, 1992).

+OM culture medium, with the highest germination rate, contains 2 mg/L activated charcoal in addition to organic nitrogen source unlike –OM culture medium. Probably activated charcoal provides a dark environment to seeds and increases the germination rate accordingly. Paek and Murthy (1977) have reported that activated charcoal prevents light entry to the environment and increases rooting. Moreover, addition of activated charcoal increases and keeps constant the level of pH of the culture medium and encourages growth by increasing nitrogen uptake (Eymar et al., 2000). The promotional effects of activated charcoal on morphogenesis may be also mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, phenolic exudation, and brown exudate accumulation (Thomas, 2008).

The germinated seeds of the orchid are turned into structures called protocorms, which form the parts of roots and shoots. The protocorm formation also depends on the types of culture medium used. In our study, the highest rate of protocorm formation in *S. vomeracea* was obtained on the +OM culture medium (75.54%). This protocorm formation can also be accepted as the rate of seed germination; the rate is an indicative value showing the amount of germinated seeds giving plantlets. Unless there is no adverse condition, almost all of the protocorms can be converted into plants (as in the present study). Moreover, very rarely some of the protocorms raised more than one plantlet.

Table 2. Effects of culture medium on the germination parameters.

	KCM	LM	–OM	+OM
Germination (%)	27.95 ± 2.4d	23.11 ± 1.9c	43.11 ± 1.5b	73.74 ± 1.6a
Protocorm formation (%)	52.22 ± 2.9c	53.70 ± 3.1c	61.22 ± 1.0b	75.54 ± 1.4a
Germination time (days)	60	60	30	20

–OM: Orchimax without activated charcoal, KCM: Knudson C orchid medium, LM: Lindemann orchid medium, +OM: Orchimax including activated charcoal. Same letters in each row were not significantly different at $P < 0.05$ (Duncan's multiple range test). Means are given with standard deviations.

It is known that orchids establish a symbiotic relationship with mycorrhizal fungi in order to germinate in nature. As indicated in the literature, symbiotic fungi produce cytokinins for the germination of seeds (Crafts and Miller, 1974). In our study, in addition to the culture medium effect on the in vitro germination of *S. vomeracea*, the effects of cytokinins and auxins were also determined (Figure 1). Higher concentrations of ZEA, 6-BA, IBA, and IAA exhibited a positive effect on germination, whereas those of TDZ were adverse and 2.4-D concentration has no effect on germination at all. The highest germination rates were observed in media containing 1.0 and 2.0 mg/L 6-BA as 83.81% and 84.03%, respectively. According to the results, the cytokinins may be regarded as more effective than auxins on germination of *S. vomeracea* seeds.

Godo et al. (2010) reported that auxins also have some effects, not as strong as cytokinins, on the germination of terrestrial orchids in culture media. Cytokinins stimulated the germination of seeds by eliminating the inhibitory effects of abscisic acid on germination, as reported before (Black et al., 1974).

Protocorm formation was positively influenced by increasing concentrations of all tested PGRs except the 2,4-D (Figure 2). The highest rate of protocorm

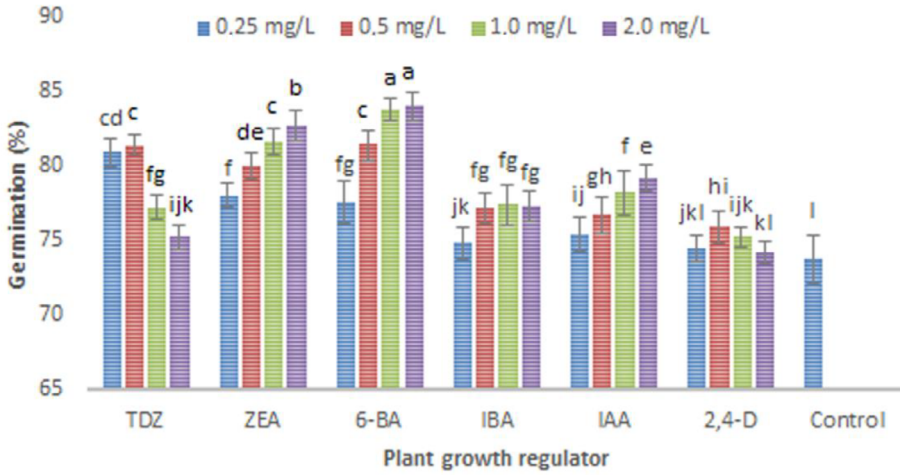

Figure 1. Effects of plant growth regulators on the germination rate of *S. vomeracea* seeds on Orchimax including activated charcoal medium after 2 months. Same letters were not significantly different at $P < 0.05$ (Duncan's multiple range test) and standard deviations are shown with bars.

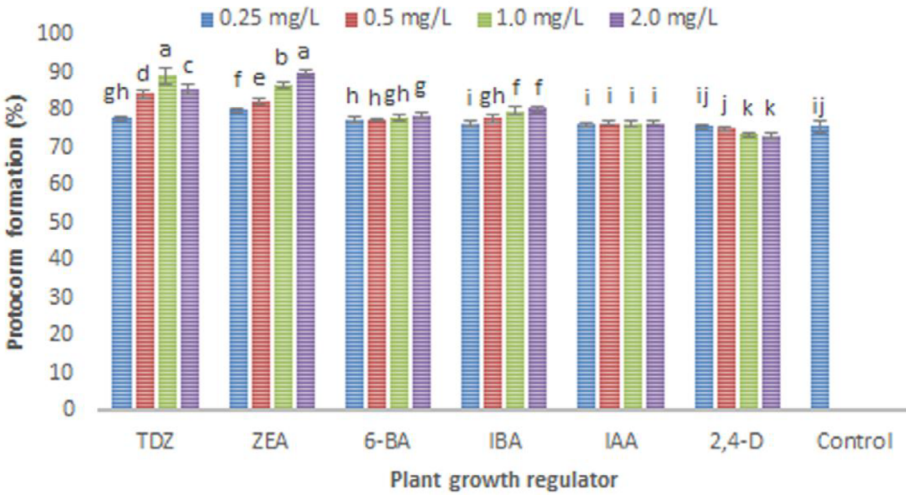

Figure 2. Effects of plant growth regulators on the protocorms formation of *S. vomeracea* on Orchimax including activated charcoal medium after 3 months. Same letters were not significantly different at $P < 0.05$ (Duncan's multiple range test) and standard deviations are shown with bars.

formation (89.91%) was observed in medium containing 2.0 mg/L ZEA. Bektaş et al. (2013) reported that media supplemented with PGRs stimulate protocorm formation without giving the type or concentrations of PGRs used.

As a result, seedlings were produced by using protocorms obtained from in vitro germinated seeds. The effects of different concentrations of cytokinins (TDZ, ZEA, KIN, 6-BA, and 2-iP) and auxins (IBA, IAA, and 2.4-D) on shoot and root length and number of leaves and roots were also determined and are shown in Tables 3 and 4, respectively. Based on these data, TDZ, ZEA, and IBA affected shoot elongation. Among the cytokinins and cytokinin-like PRGs employed, the highest shoot elongation was obtained (42.88 mm) in medium containing 0.25 mg/L TDZ. As reported before, 1.0 to 2.0 mg/L cytokinin is sufficient in most systems and higher levels have a tendency to increase the adventitious shoot formation, and synthetic auxins are more preferable since IAA, which is a natural auxin, is not very stable in nature. The ratios used in the reproduction of shoots are determined as 0.1 to 1.0 mg/L (Skoog and Miller, 1957; Werbrouck and Debergh, 1994).

According to the results, the culture medium containing 2.0 mg/L IBA, with an average of 3.8 leaves, was found to be more effective in the formation of leaves. In previous studies, no report was found stating that IBA increases the formation of leaves in plants. However, it is known that stimulating shoot formation and inhibiting abscission of leaves are among the physiological effects of plant growth regulators of the cytokinin group (Gaspar et al., 1996; Haberer and Kieber, 2002).

The effects of different concentrations of cytokinins and auxins on the length and number of roots of *S. vomeracea* were determined. Medium containing 2.0 mg/L IBA is the

Table 3. Effects of cytokinins on the shoot (60 days), root (90 days), tuber formation, and glucomannan content (11 months) on Orchimax including activated charcoal medium.

PGRs	Concentrations (mg/L)	Shoot elongation (mm)	Root length (mm)	Root number	Leaf number	Tuber formation	Glucomannan content (%)
TDZ	0.25	42.88 ± 1.8a	34.15 ± 0.9b	3.73 def	3.18 ab	36.7	40.8 ± 0.4
	0.5	31.94 ± 1.7c	32.54 ± 0.9c	3.66 def	2.90 ab	36.6	39.8 ± 0.5
	1.0	24.46 ± 1.5fg	30.64 ± 0.8d	3.62 def	2.81 ab	28.6	22.3 ± 0.5
	2.0	19.58 ± 1.7hijk	28.90 ± 1.1e	3.56 def	2.72 b	-*	-*
ZEA	0.25	20.25 ± 1.6hij	37.50 ± 0.9a	3.93 cde	2.90 ab	-	-
	0.5	24.18 ± 1.6g	37.80 ± 0.9a	4.33 bc	3.00 ab	53.5	35.8 ± 0.7
	1.0	28.83 ± 1.7d	37.66 ± 0.7a	4.62 b	3.27 ab	55.5	41.0 ± 0.6
	2.0	34.15 ± 1.3b	37.78 ± 0.5a	5.18 a	3.36a	57.3	44.6 ± 0.3
KIN	0.25	19.08 ± 1.1jk	23.65 ± 0.9h	3.33 ef	2.86 ab	-	-
	0.5	19.88 ± 1.2hijk	23.61 ± 1.3h	3.26 f	2.92 ab	-	-
	1.0	20.19 ± 1.5hij	23.51 ± 1.1h	3.25 f	2.93 ab	39.1	22.8 ± 0.9
	2.0	20.64 ± 1.7hi	23.48 ± 0.7h	3.25 f	3.00 ab	35.3	25.5 ± 0.5
6-BA	0.25	24.59 ± 1.6fg	25.21 ± 1.1f	3.66 def	2.86 ab	36.3	28.4 ± 1.1
	0.5	25.36 ± 1.5efg	24.60 ± 1.2fg	3.73 def	2.87 ab	39.4	30.4 ± 1.0
	1.0	25.68 ± 1.2ef	24.43 ± 1.1g	3.81 cdef	2.93 ab	39.8	33.1 ± 0.6
	2.0	26.12 ± 1.4e	24.25 ± 0.8gh	4.06 cd	2.93 ab	-	-
2-iP	0.25	18.80 ± 1.2jk	18.50 ± 1.1i	2.46 g	2.83 ab	-	-
	0.5	19.37 ± 1.5ijk	18.13 ± 0.7i	2.46 g	2.91 ab	-	-
	1.0	20.27 ± 1.9hij	17.99 ± 0.8j	2.43 g	2.92 ab	-	-
	2.0	20.96 ± 2.2h	17.29 ± 0.9j	2.43 g	2.90 ab	29.2	23.1 ± 0.3
Cont.		18.43 ± 1.4k	18.52 ± 1.2i	2.00 g	2.69 b	-	-

+OM without any plant growth regulators was used as control. Shoot length and leaf number (60 days), root length and number (90 days), and tuber formation rates (11 months) were measured days after sowing of the protocorms on +OM. Same letters in each column were not significantly different at $P < 0.05$ (Duncan's multiple range test). Means are given with standard deviations.
* Tuber formation was not observed and glucomannan content was not identified

Table 4. Effects of auxines on the shoot, root, tuber formation, and glucomannan content on Orchimax including activated charcoal medium.

PGRs	Concentrations (mg/L)	Shoot elongation (mm)	Root length (mm)	Root number	Leaf number	Tuber formation	Glucomannan content (%)
IBA	0.25	22.71 ± 0.4f	28.95 ± 0.6f	4.8abc	3.2bcde	-*	-*
	0.5	28.69 ± 0.4d	32.96 ± 0.6d	5.0ab	3.6abc	36.8	33.8 ± 1.0
	1.0	31.73 ± 0.5ab	36.04 ± 0.5b	5.2a	3.7ab	37.4	33.6 ± 0.5
	2.0	31.92 ± 0.7a	38.08 ± 0.9a	5.2a	3.8a	39.6	35.0 ± 0.3
IAA	0.25	19.86 ± 0.7h	27.10 ± 0.5g	4.5abcd	3.3abcde	-	-
	0.5	24.41 ± 0.7e	31.38 ± 0.6e	4.6abcd	3.4abcde	-	-
	1.0	29.16 ± 0.6c	35.22 ± 0.5c	4.9ab	3.5abcde	35.6	29.8 ± 0.7
	2.0	31.44 ± 0.5b	35.90 ± 0.7b	5.0ab	3.6abcd	38.0	32.9 ± 0.6
2.4-D	0.25	19.02 ± 0.8h	18.89 ± 0.4j	4.1d	3.0e	-	-
	0.5	19.13 ± 0.7h	23.24 ± 0.5i	4.2cd	3.1de	-	-
	1.0	22.18 ± 0.5g	26.26 ± 0.7h	4.4bcd	3.2cde	-	-
	2.0	22.20 ± 0.5g	27.25 ± 0.5g	4.5abcd	3.2cde	-	-
Cont.		18.43 ± 0.2i	18.52 ± 1.2j	2.00 e	2.69 f	-	-

+OM without any plant growth regulators was used as control. Shoot length and leaf number (60 days), root length and number (90 days), and tuber formation rates (11 months) were measured days after sowing of the protocorms on +OM. Same letters in each column were not significantly different at $P < 0.05$ (Duncan's multiple range test). Means are given with standard deviations.
* Tuber formation was not observed and glucomannan content was not identified

most appropriate one for root elongation. The average root elongation was 38.08 mm in this medium. The highest average number of roots was found in media containing 1.0 and 2.0 mg/L IBA as 5.2 units. Considering the results obtained, it is seen that auxins give better results in terms of root length and number compared to cytokinins. In the literature, auxins are needed in the culture medium for root formation and there are also several studies indicating that auxins stimulate the formation of roots (Bhojwani and Razdan, 1983; Preece and Sutter, 1991; Aktar et al., 2007; Díaz and Álvarez, 2009).

3.2. Tuberization and glucomannan content of tubers

Another parameter investigated is to find the effects of auxins and cytokinins on tuber formation of orchid species. First of all, it should be emphasized that *S. vomeracea* could form tubers in culture medium after just 11 months. The highest rate of tuber formation (57.3%) was observed in a culture medium with 2.0 mg/L ZEA. Based on these results, 1.0 and 2.0 mg/L concentrations of ZEA are more effective in tuber formation of *S. vomeracea* compared to other plant growth regulators. Similar results were reported previously by Pedroso and Pais (1992). Researchers investigated the formation of minitubers in *Orchis papilionacea* L. on MS culture medium and determined that the most effective plant growth regulator was ZEA.

Cytokinins (especially ZEA and TDZ) were found to be more efficient compared to auxins in terms of tuber formation. It has been observed that 2-iP (except 2.0 mg/L concentration) cytokinins were ineffective for the formation of *S. vomeracea* tubers. According to previous studies, some available information indicates that cytokinins and gibberellins are the most important chemicals regulating the formation of tubers (Jackson, 1999; Sarkar, 2008). Gibberellins inhibit the formation of tubers, and cytokinins stimulate tuber formation by triggering cell division (Vreugdenhil and Sergeeva, 1999; Fernie and Willmitzer, 2001).

The glucomannan content of the in vitro propagated plants is a very important parameter as such an investigation has not been taken into account before. The glucomannan contents of tubers obtained from cultured plants growing in the presence of different plant growth regulators show great variability. The highest amount of glucomannan (44.6%) in *S. vomeracea* was obtained from tubers formed in the medium containing 2.0 mg/L ZEA. The amounts of glucomannan stored in tubers of *S. vomeracea* seedlings grown in basal medium with 0.25 and 0.5 mg/L TDZ and 1.0 and 2.0 mg/L ZEA were determined. They accumulated higher amount of glucomannan than those obtained from their natural sources.

There are several studies concerning the glucomannan contents of various orchid plants, including *S. vomeracea* (Farhoosh and Riazi, 2007; Tekinsen and Güner, 2010; Chua et al., 2012). According to Tekinsen and Güner (2010), the glucomannan content of naturally growing *S. vomeracea* subsp. *orientalis* was 44.8%. In our study, the glucomannan content of naturally growing *S. vomeracea* was determined as 37.4% by using the phenol–sulfuric acid method while those of micropropagated plants mentioned here were the range of 22.3%–44.6%.

3.3. Synthetic seed production and acclimatization

Uncontrolled and excessive collection of orchid tubers, as they have economic importance, and dismantling of the plants without allowing the maturation of seeds result in a decrease in the number of these species and even cause some to remain in danger of extinction.

Protocorm-like bodies of *S. vomeracea* were established by using Orchimax basal media supplemented with either TDZ (0.1, 0.25, 0.5, 1.0, and 2.0 mg/L) alone or in combination with 1.0 mg/L IBA. Although the formation of protocorm-like structures (Figure 3A) was observed in all media employed, the formation rate as well as amounts of PLBs varied depending upon the media PGR profiles (Table 5). The highest PLB formation rate (96%) was obtained from the medium containing a combination of IBA (1.0 mg/L) and TDZ (0.5 and/or 2.0 mg/L) where the number of PLB per explant was 14.9 and 14.4 on average, respectively.

The positive effects of TDZ on the regeneration as well as PLB formation from orchid plants have been described elsewhere (Park et al., 2003; Singh et al., 2003; Roy et al., 2007). As reported, lower concentrations of TDZ stimulate the formation of multiple shoots of *Cajanus cajan*, while higher concentrations stimulate somatic embryogenesis (Singh et al., 2003). Using the combination of TDZ and NAA increased the formation of somatic embryos observed in *Oncidium* Sw. (Chen and Chang, 2000).

Synthetic seeds of *S. vomeracea* (Figure 3B) were produced by encapsulating PLBs with 3% sodium alginate and solidification in 75 mM of $CaCl_2$. According to the previous reports, amounts of sodium alginate and $CaCl_2$ used for preparation of the encapsulation matrix are 3%–4% and 75–100 mm, respectively (Daud et al., 2008; Geetha et al., 2009; Sarmah et al., 2010; Nor Asmah et al., 2011). The encapsulation matrix prepared by the aforesaid procedure provides earlier germination of synthetic seeds with higher rates (Saiprasad and Polisetty, 2003).

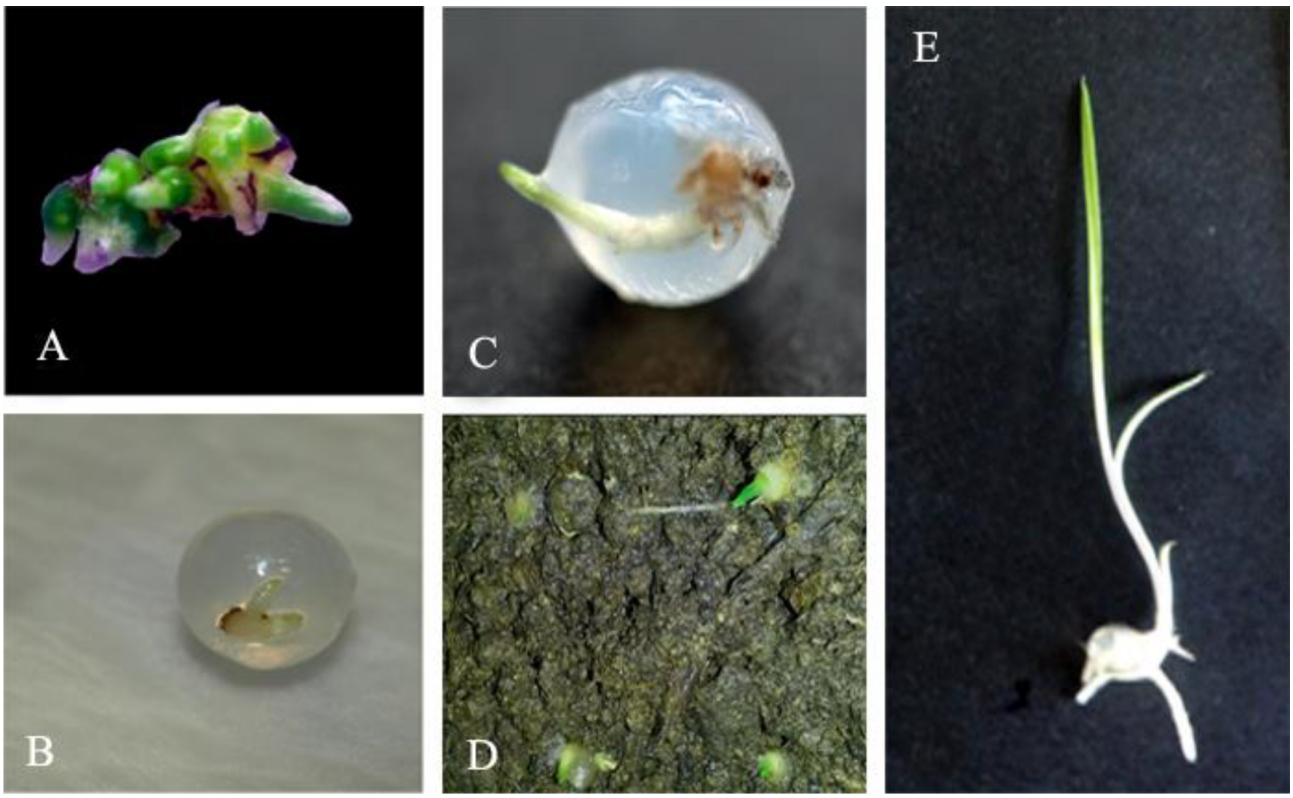

Figure 3. Evaluation of synthetic seeds of *S. vomeracea* A: Protocorm-like bodies of *S. vomeracea*, B: Encapsulated protocorm-like bodies of *S. vomeracea*, C: Germination of synthetic seeds, D: Germination of synthetic seeds on sterilized peat medium, E: Plantlet from in vitro germinated synthetic seed.

Table 5. Effects of plant growth regulators on the formation of protocorm-like bodies on Orchimax including activated charcoal medium after 3 months.

PGRs	Concentration (mg/L)	PLB formation (%)	Number of PLB per explant
TDZ	0.1	6	4.0g
	0.25	26	5.6f
	0.5	88	9.7d
	1.0	48	14.7a
	2.0	81	13.5c
TDZ + IBA	0.1 + 1.0	16	4.3g
	0.25 + 1.0	66	6.8e
	0.5 + 1.0	96	14.9a
	1.0 + 1.0	88	14.0bc
	2.0 + 1.0	96	14.4ab

The control group (+OM without any plant growth regulators) showed no protocorm-like bodies formation. Same letters in each column were not significantly different at $P < 0.05$ (Duncan's multiple range test).

The germination ability of synthetic seeds under in vitro and soil conditions are indicated in Figures 3C and 3D. The germination percentage in the former case was around 80%, while that of the latter was 75%. The rates of germinated synthetic seeds giving rise to seedlings (Figure 3E) were 100% on culture media and 60% on peat substrate. In the nonsterilized peats, synthetic seeds lost their viability as a result of fungal contamination. The germination rates of synthetic seeds from protocorm-like structures were higher than those of zygotic embryos (natural seeds) germinated under in vitro conditions. In addition, all synthetic seeds germinated on the culture media gave seedlings. However, with the sterilized peat medium, some of the germinated seeds failed to form seedlings. Saiprasad and Polisetty (2003) have reported that all synthetic seeds of *Dendrobium*, *Oncidium*, and *Cattleya* orchids germinated on MS medium. In the same study, the researchers successfully germinated synthetic seeds of three species in pots containing either charcoal pieces or charcoal and briquette pieces together (Saiprasad and Polisetty, 2003).

Successful results were obtained when the in vitro growing seedlings were gradually transferred to the sterilized peat medium (Figure 4). Seedlings (9%) lost their vitality after the lids were reopened (after 30 days) and development of the seedlings was maintained in this way. At the end of 90 days, the survival rate of the seedlings was determined as 91%.

This study reports on procedures for the effective production of *S. vomeracea* by using plant tissue culture techniques such as in vitro asymbiotic germination that can be used for the conservation and propagation of

Figure 4. Plantlet development of *S. vomeracea* 2 months after planting in sterilized peat medium.

endangered or threatened orchid species. Further research on adaptation of in vitro grown seedlings and synthetic seeds to agricultural fields or natural environments of these species is recommended. These studies are useful for conservation of endangered or threatened orchid species.

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