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Direct shoot regeneration from nodes of *Phalaenopsis* orchids

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ABSTRACT

Nodes with dormant buds from flower stalks of orchid *Phalaenopsis* sp. were plated on six culture media. The composition of the culture media affected the induction, regeneration, number and form of *Phalaenopsis* regenerants. We managed to obtain direct shoot regeneration without callus formation on all media except for medium F. Medium A, supplemented with 2 mg/l of 6-benzylaminopurine (BAP) and 0.5 mg/l of α–naphtalenacetic acid (NAA) was found to be the most appropriate of all the media used for rapid micropropagation of a large number of vegetative shoots (multiplication factor 8.35 per node) without roots 160 days after inoculation. We found medium B, supplemented with 4.41 mg/l BAP and 1 mg/l NAA less appropriate for vegetative shoot production (multiplication factor 2.08 per node) also because the regenerants ceased to elongate. Medium C, containing BAP (2 mg/l) and a lower nitrogen content showed the highest multiplication rate (0.54 per node) for generative regenerants formation. Vegetative regenerants with a generative shoot also formed. This type of regenerant could be of major commercial interest, since period until flowering would be much shortened. Dormant bud induction was poor on medium E (23% regeneration rate) and there was no regeneration on medium F. Regenerants with well-developed roots on medium D (no hormones) most resembled the adult plants, but the vegetative multiplication rate was lower (1.18 per node). Most vegetative regenerants were ready for acclimatization after 90 days of subcultivation on medium D, which also proved to be a successful subcultivation medium for regenerants formed on media A, B and C.

Key words: *Phalaenopsis,* monopodial orchids, culture media composition, flower stalks, *in vitro* culture, micropropagation, vegetative regenerants, generative regenerants

IZVLEČEK

DIREKTNA REGENERACIJA POGANJKOV IZ NODIJEV ORHIDEJ *PHALAENOPSIS*

Nodije z dormantnimi brsti s cvetnih poganjkov orhidej *Phalaenopsis* sp. smo inokulirali na šest različnih gojišč. Sestava gojišč je vplivala na indukcijo, regeneracijo in obliko regenerantov *Phalaenopsis*. Na vseh gojiščih z izjemo gojišča F je bila direktna regeneracija

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poganjkov brez oblikovanja kalusa. Ugotovili smo, da je gojišče A (2 mg/l BAP, 0,5 mg/l NAA) najprimernejše gojišče za hitro mikropropagacijo velikega števila vegetativnih poganjkov (multiplikacijski faktor 8,35 na nodij) brez korenin 160 dni po inokulaciji. Gojišče B (4,41 mg/l BAP, 1 mg/l NAA) je bilo manj primerno za nastanek vegetativnih regenerantov (multiplikacijski faktor 2,08 na nodij) tudi zaradi neizdolževanja regenerantov. Generativnih regenerantov je bilo največ (multiplikacijski faktor 0,54 na nodij) na gojišču C (2 mg/l BAP in nižja vsebnost dušika). Nastalo je tudi nekaj vegetativnih regenerantov z generativnim poganjkom, ki bi bili komercialno zelo zanimivi zaradi skrajšanega časovnega obdobja potrebnega do cvetenja. Indukcija dormantnih brstov je bila nizka na gojišču E (23% regeneracija) oziroma sploh ni bilo regeneracije na gojišču F. Regeneranti na gojišču D (brez hormonov) z dobro razvitimi koreninami so bili najbolj podobni odraslim rastlinam, vendar je bil multiplikacijski faktor za vegetativne poganjke nižji (1,18 na nodij). Večina vegetativnih regenerantov je bila primerna za aklimatizacijo že po 90-ih dneh subkultivacije na gojišču D. Gojišče D se je izkazalo tudi kot uspešno subkultivacijko gojišče regenerantov z gojišč A, B in C.

Ključne besede: *Phalaenopsis*, monopodialne orhideje, sestava gojišč, cvetni poganjki, *in vitro* kultura, mikropropagacija, vegetativni regeneranti, generativni regeneranti

1 INTRODUCTION

Phalaenopsis hybrids are a result of intensive breeding of plants of increasing market value as cut flowers and even more as potted plants. Because of the large, colourful and durable flowers, as well as their adaptability to room conditions, they are the most popular orchid genus in the horticultural industry. The duration and intensity of flowering depends not only on the genotype but also the breeding technology, which greatly influences the plants' vitality. *Phalaenopsis* is a monopodial epiphytic orchid, which is difficult to propagate vegetatively. The characteristics of seedlings are not uniform, and propagation through tissue culture has been desired.

Several tissue culture techniques have been developed for *Phalaenopsis* orchids, including the culture of flower stalks with axillary buds, meristems, flower stalks explants, internodal segments of flower stalks (Arditti and Ernst, 1993; Park et al., 1996), leaf segments (Park et al., 2002; Ishii et al., 1998) and root tips (Arditti and Ernst, 1993; Ichihashi, 1997). Due to difficulties in regeneration to protocorms and then to plants, all the procedures mentioned above have been inadequate for meeting commercial needs for vegetative propagation. Some of these methods gave a lot of protocorms, but these developed slowly or poorly to vital plants. Tokuhara and Mii (1993) reported that the combination and appropriate concentration of hormones α–naphtalenacetic acid (NAA) and 6-benzylaminopurine (BAP) and the composition of macro- and microelements in the culture medium were of key importance for micropropagation of *Phalaenopsis* on a commercial scale.

Some authors have reported frequent callus formation as an intermediary phase just prior to somatic embryogenesis or regeneration to protocorms (Tisserat and Jones, 1999; Ishii et al., 1998). Direct regeneration without undesirable callus formation shortens the time period needed for regeneration and reduces the possibility of the occurrence of somaclonal variability. In the present study, different culture media for direct shoot regeneration from nodes of *Phalaenopsis* flower stalks were tested in order to develop an efficient and rapid propagation method for these orchids.

2 MATERIALS AND METHODS

2.1 Plant material

Eight hybrids of *Phalaenopsis* sp. in the first blossom stage, with three to four open flowers, were used as source material for culture. Explants from *Phalaenopsis* plants with one to three flower stalks and three to nine nodes with dormant buds were randomly placed onto six different culture media. Nodes with dormant buds originating from a single flower stalk were inoculated on the same medium in order to investigate whether their regeneration capacity is influenced by the location of the dormant bud on the flower stalk, from the top down to the base.

Table 1: Composition of culture media for vegetative propagation of *Phalaenopsis* orchids from nodes.

Components	Culture medium					
	A	**B**	**C**	**D**	**E**	**F**
Macro elements (mg/l)						
KH_2PO_4	85	170	-	-	250	170
NH_4NO_3	825	1650	-	-	-	1650
$(NH_4)_2SO_4$	-	-	135	135	500	-
$MgSO_4$	90.35					
$MgSO_4 \times 7H_2O$		370	250	250	250	370
KNO_3	950	1900	2500	2500	525	1900
$CaCl_2$	166	332.2	-	-	-	-
$CaCl_2 \times 2H_2O$	-	440	150	150	-	440
$NaH_2PO_4 \times H_2O$	-	-	150	150	-	-
$FeSO_4 \times 7\ H_2O$	27.85	27.8	-	-	27.8	27.8
$Ca_3(PO_4)_2$		-	-	-	200	-
Microelements (mg/l)						
H_3BO_3	3.1	6.2	6.2	2.6	-	6.2
$MnSO_4 \times H_2O$	8.45	-	-	-	-	-
$MnSO_4 \times 4H_2O$	-	22.3	22.3	22.3	5	22.3
$ZnSO_4 \times 7H_2O$	5.3	8.6	8.6	8.6	-	8.6
KJ	0.415	0.83	0.83	0.83	-	0.83
$CuSO_4 \times 5H_2O$	0.0125	0.025	0.025	0.025	-	0.025
$CoCl_2 \times 6H_2O$	0.0125	0.025	0.025	0.025	-	0.025
$Na_2MoO_4x2H_20$	0.125	0.25	0.25	0.25	-	0.25
Na_2Fe-EDTA	37.24	37.2	25	25	37.3	37.3
Carbon hydrate (g/l)						
Sucrose	20	3	25	25	20	30
Organic compounds, vitamins, hormones (mg/l)						
Myo-Inositol	100	100	-	-	-	100
Casein hydrate	-	100	-	-	-	-
Peptone	2000	-	-	-	-	-
MES	1000	-	-	-	-	-
Pyridoxine	0.5	1	-	-	-	-
Thiamine	1	1	-	-	-	0.1
Nicotinic acid	0.5	1	-	-	-	-
BAP	2	4.41	2	-	-	-
NAA	0.5	1	-	-	-	-
IAA	-	-	-	-	-	1.9
KIN	-	-	-	-	-	2.2
Gelling agent (g/l) and pH value						
Gellan gum	2.6	2.6	2.6	2.6	2.6	2.6
pH	5.2	5.7	5.2	5.4	5.5	5.2

EDTA - ethylenediaminetetraacetic acid, MES - morpholino ethane sulfonic acid, BAP - 6-benzylaminopurine, NAA - α–naphtalenacetic acid, IAA - indolacetic acid, KIN - kinetin.

2.2 Culture media composition

Culture media for vegetative reproduction of *Phalaenopsis* orchids were chosen from among the most efficient available media according to authors in the literature. Some of the components were slightly modified for the special needs of our experiment . The composition of medium A was the same as P 6793 (Sigma) commercial medium. Medium B contained macro and microelements of MS medium (Murashige and Skoog, 1962); other components used were as described in Tisserat and Jones (1999). Medium D was composed of B5 medium's (Gamborg et al., 1968) macro elements, MS medium's microelements and other components from the medium described by Hinnen et al. (1989) but without banana homogenate. Medium C was the same as medium D except for the addition of hormone BAP (6-benzylaminopurine). Media E and F were composed according to Arditti and Ernst (1993) except for the gelling agent (medium E) alone or gelling agent and carbon source (medium F) combined (sucrose instead of glucose). The gelling agent in all media was gellan gum instead of agar. The composition of all culture media used is described in Table 1.

2.3 Culture initiation

Flower stalks were cut into 50 – 60 mm long single-node cuttings with parts of internodes, each node holding one dormant lateral bud. Bracts at the nodes were carefully removed just before sterilization. The cuttings were surface-sterilized with 16.6 g/l dichloroisocyanuric acid Na_2 salt with the addition of a few drops of Tween 20 for 10 min, and rinsed three times with sterilized water. Approximately 10-15 mm of damaged internodal tissue on both sides of the sterilized segments was cut off. Segments, 30 – 35 mm in size, were then cultivated on randomly chosen culture media (A-F) in glass jars. Buds originating from a single plant were inoculated on the same medium, where possible, in order to identify possible differences in regeneration capacity between individual lateral buds from the same flower stalk. The cuttings, and later on the regenerants, were grown in a climatic chamber under the same conditions of a 16/8 h photoperiod at 24 ± 1 °C, and illumination of 34 $\mu E/m^2s$. The explants were subcultured onto the same medium every 30 days, except for explants on medium E (just once) and medium F (no sub-cultivation) due to poor regeneration response. The induction of dormant buds to vegetative ones, generative regenerants and protocorms was also evaluated every 30 days.

The results of the regeneration experiment were statistically analyzed with Duncan's multiple range test at $P \le 0.05$ (Duncan, 1955) to evaluate differences in regeneration capacity for producing vegetative and generative regenerants among different culture media.

3 RESULTS AND DISCUSSION

3.1 Sterilization of flower stalk segments

The sterilization procedure with 16.6 g/l dichloroisocyanuric acid Na_2 salt proved to be successful, since all of the cuttings treated were undamaged and only 4% of them became infected. The damaged internodal tissue on both sides of the sterilized segments was removed. The remaining 10-15 mm of internodal tissue on the explants prevented nodes with buds from drying up.

3.2 Influence of media composition on dormant bud regeneration

In our experiment, nodes with dormant buds were inoculated onto six culture media. The composition and pH value of the culture medium affected the induction, regeneration, number and form of *Phalaenopsis* regenerants. Dormant buds enlarged and induction was triggered in a period of 10 to 30 days, regardless of the medium used, which approximately corresponds with data in literature (Tisserat and Jones, 1999). Buds were subcultured onto the same medium in order to investigate

differences between individual media after induction and regeneration. The most successful culture media were media A, B, C and D, with 100% regeneration of the dormant buds. Medium E (23% regeneration rate) and medium F (no regeneration) were less successful, the majority of the dormant buds not even being induced, and only in a few cases could we find regeneration initiation or enlarged buds.

Table 2: Formation of vegetative and generative regenerants from dormant buds of *Phalaenopsis* orchids on six culture media 160 days after inoculation.

Culture medium	Plant explants	Vegetative regenerants	Multiplication factor*	Generative regenerants	Multiplication factor*
A	14	117	8.35a	2	0.14c
B	12	25	2.08c	0	0
C	11	31	2.82b	6	0.54a
D	11	13	1.18d	2	0.18b
E	13	3	0.23e	0	0
F	11	0	0	0	0

* Multiplication factors followed by different letters are significantly different according to the Duncan's test ($P \leq 0.05$).

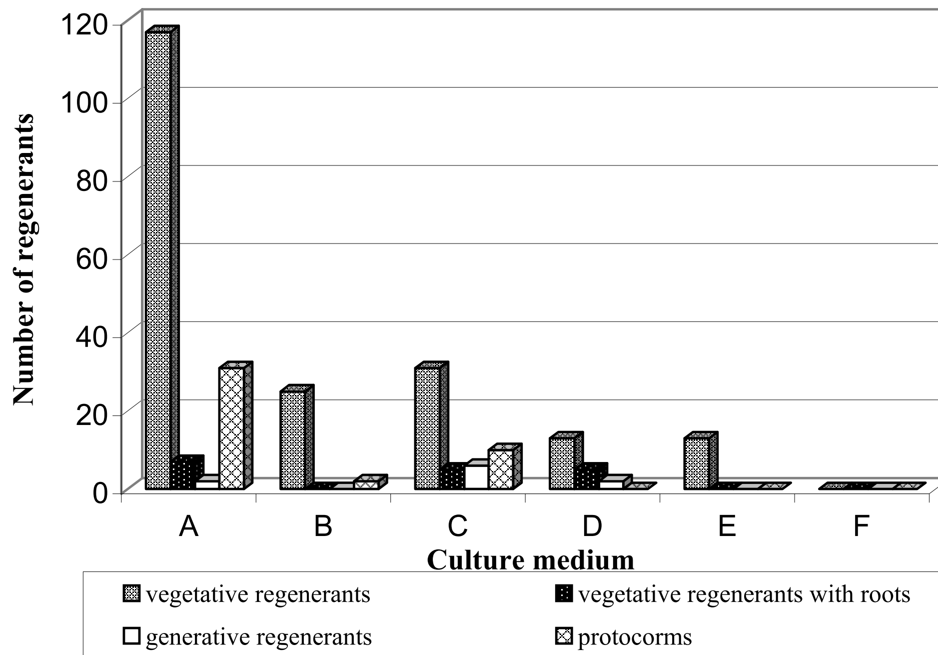

Fig. 1: Effect of different culture media (A-F) on *Phalaenopsis* dormant bud regeneration rate 160 days after inoculation.

The commercial medium A was found to be the best choice as regards vegetative regenerants production. One hundred and sixty days after inoculation, 117 vegetative regenerants (Table 2; Fig. 1) with two to five leaves formed and seven of them had two to four roots. The multiplication factor of the vegetative regenerants was 8.35 (Table 2). The regenerants were somewhat smaller but there were more of them in

clusters (Fig. 2a, 2e) in comparison with other media. Every 30 days an average of 20 vegetative regenerants formed on clusters and also on the excised bases of the explants. One hunded and sixty days after inoculation, only two generative regenerants (Table 2), as well as 31 protocorms, had developed (Fig. 1). The two generative regenerants arose directly from inoculated dormant buds. In later subcultivations, only vegetative regenerants evolved from newly formed protocorms (Fig. 2f).

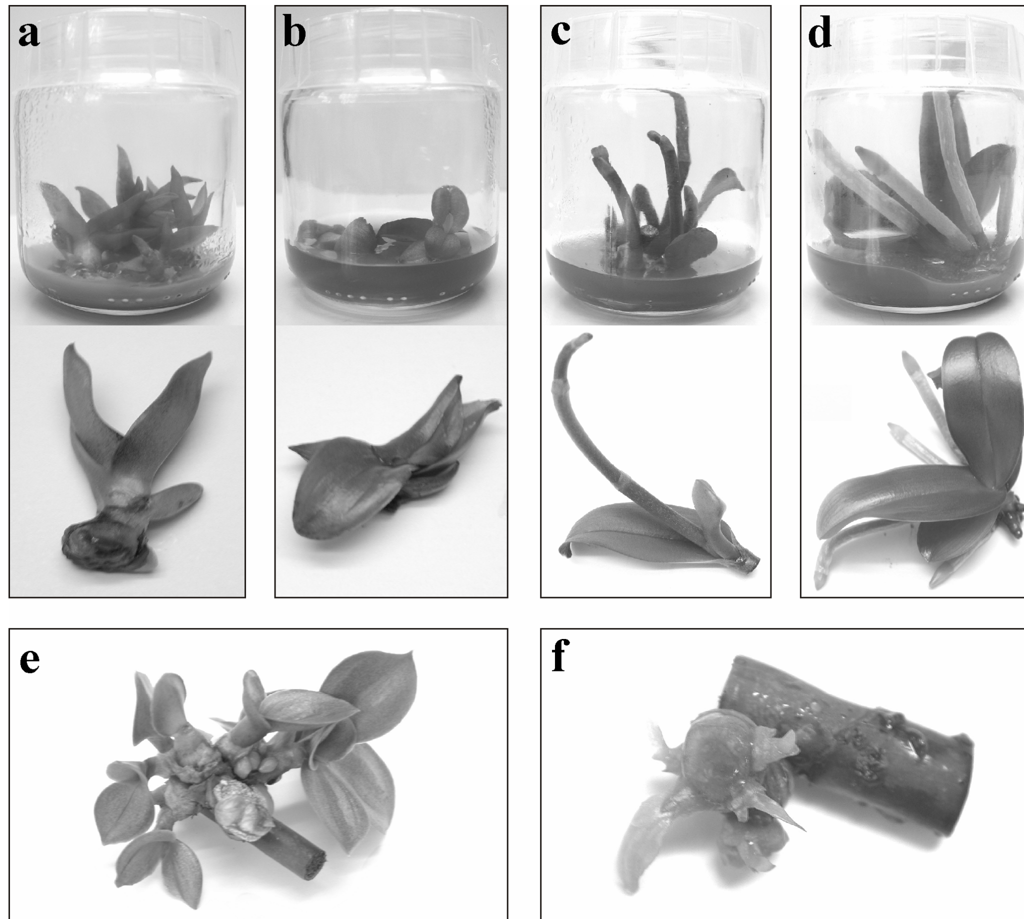

Fig. 2: Direct shoot regeneration from nodal explants of *Phaleonopsis* flower stalks on different culture media: a) vegetative regenerants with enlarged basal part on medium A, b) shorter vegetative regenerants on medium B, c) vegetative regenerants with generative shoots on medium C, d) vegetative regenerants with roots on medium D; e) cluster of vegetative regenerants emerging from nodal explant; f) development of vegetative regenerants from protocorms.

Dormant buds on medium B just enlarged in the 30-day period and regeneration started 10 to 30 days later than on medium A. Only 25 vegetative regenerants (Table 2; Fig. 1) with two to four leaves and no roots had formed 160 days after inoculation (Fig. 2b). The multiplication factor of the vegetative regenerants was 2.08 (Table 2), which is statistically significantly less ($P \leq 0.05$) than with the medium A. There was no generative regenerants development even 160 days after inoculation.

On medium C, 31 vegetative regenerants (Table 2; Fig. 1) with two to four leaves developed, five of them with roots. The multiplication factor of 2.82 (Table 2) shows that medium C is statistically significantly ($P \leq 0.05$) less appropriate than medium A but better than medium B. Six generative shoots also emerged from vegetative regenerants (Table 2; Fig. 1, 2c) and a cluster of more than ten protocorms formed.

Only 13 vegetative regenerants (Table 2; Fig. 1) with two to four leaves, five of which had one to four roots, developed 160 days after inoculation on medium D. The vegetative regenerants on this medium most resembled adult *Phalaenopsis* plants and had more vital roots (Fig. 2d). Most of them were ready for acclimatization after only 90 days. The multiplication factor of 1.18 (Table 2) indicates statistically significantly ($P \leq 0.05$) lower efficiency than media A, B and C. Two generative regenerants evolved (Table 2).

Arditti and Ernst (1993) recommended media E and F as very suitable culture media for vegetative propagation of *Phalaenopsis* orchids. In our case this turned to be just the opposite, since the other media used gave a 100% regeneration rate. On medium E (23% regeneration rate) and medium F (no regeneration), only induction of dormant buds occurred, some of which enlarged a bit, then the chlorophyll degraded and they finally died. The growth of the vegetative regenerants that formed on medium E rather lagged behind regenerants on the other media. The multiplication factor of vegetative regenerants was only 0.23 (Table 2), which is statistically significantly ($P \leq 0.05$) lower than media A, B, C and D. There was no generative regenerants development on medium E (Table 2).

Interestingly, there were major differences among the culture media used between regeneration capacities and the morphology of the plantlets, despite the fact that their composition did not vary much (Table 1). The morphology of regenerants on media C and D most resembled the adult plants (Fig. 2c, 2d), probably due to the lack of ammonium nitrate and potassium phosphate and the lower amounts of iron in comparison with other media. Plantlets on medium D were also the largest and with multiple, well developed roots (Fig. 2d). Regenerants on medium B remained short and with small leaves (Fig. 2b) probably because of the highest concentration of ammonium nitrate and potassium phosphate and the lowest concentration of sucrose (3 g/l; other media 20-25 g/l) in comparison with other media. On medium A, there were many smaller plantlets in clusters without roots (Fig. 2a). The multiplication rate was the highest among the six media used (Table 2), presumably due to appropriate cytokinin (BAP – 2 mg/l) and auxin (NAA – 0.5 mg/l) content in comparison with media B (BAP 4.41 mg; NAA 1 mg/l), C (just BAP 2 mg/l), D (no hormones), E (no hormones) and F (just auxins IAA 1.9 mg/l and KIN 2.2 mg/l). Ernst (1994) reported that shoot and root development were reduced, while proliferation increased, with increasing concentrations of cytokinin TDZ (thidiazuron). Some authors have reported that the addition of NAA reduced induction and regeneration (Arditti and Ernst, 1993), others that an appropriate combination of NAA and BAP stimulated shoot formation (Tokuhara and Mii, 1993; Tisserat and Jones, 1999; Roy and Banerjee, 2003). Arditti and Ernst (1993) recommended medium F as very suitable for vegetative propagation of *Phalaenopsis* and *Cymbidium* orchids. In our study, there was no regeneration on medium F, perhaps because of an inappropriate hormone combination (just auxines) for induction of dormant buds.

We chose gellan gum instead of agar for the gelling agent in all the media used because of better germination and growth of plantlets in our previous work with *Phalaenopsis* orchids (data not shown). Ishii et al. (1998) also reported better callus induction and protocorm formation with gellan gum as the solidifier.

We found that the position of the dormant buds from top down to base of a flower stalk did not significantly affect their regeneration capacity.

Some authors (Arditti and Ernst, 1993; Tisserat and Jones, 1999) have reported frequent callus formation as an intermediary phase just before shoot regeneration. In our study, successful (in terms of shoot regeneration) media A, B, C and D enabled direct regeneration without callus formation. Media E and F proved to be inappropriate for vegetative propagation of *Phalaenopsis* orchids from dormant buds and there was also no callus formation on plant segments.

Our assessment is that none of the media studied is suitable for direct regeneration of generative or vegetative regenerants with generative shoots on a commercial scale. The multiplication factor of generative regenerants on medium C was only 0.54 (Table 2), which is statistically significantly ($P \leq 0.05$) the best among all media used. Earlier flowering is of major commercial interest, since at least three years are required from sowing seed to flower development under greenhouse conditions. The combination and concentration of plant hormones and the nitrogen and phosphorus content have a major impact on early flowering induction under *in vitro* conditions. A combined treatment of BAP, restricted nitrogen supply with phosphorus enrichment and root excision, induced a transition of *Cymbidium* shoots from vegetative to reproductive stages (Kostenyuk et al., 1999). Our medium C contained a lower total nitrogen concentration in comparison with medium A, which was the best for shoot multiplication. The formation of floral buds and the development of shoots need different levels of nitrogen and BAP. A higher BAP concentration (5 mg/l) and lower nitrogen content (4.5 mM) induced more flower buds in *Phalaenopsis* (Duan and Yazawa, 1995). Our medium C was supplemented with approximately 2.5× higher nitrogen and 2.5× lower BAP content. So medium C with slight changes in the media composition (higher BAP and lower nitrogen content) could serve for earlier flowering induction in tissue culture and later *in vivo*. Media D and A, with multiplication factors of 0.18 and 0.14, respectively, proved to be less appropriate for generative regenerant formation. There was no generative regenerant development on media B, E and F (Table 2).

3.3 Subcultivation of plant segments and regenerants

Regenerants were excised from nodes in two ways for subcultivation. With the first method, new protocorms developed on the preserved 3 – 5 mm of the basal part of regenerated dormant buds on nodes, but these protocorms later gave rise only to vegetative regenerants (Fig. 2e, 2f). In this case, the basal part represented a portion of the enlarged dormant bud, consisting of young, rapidly dividing cells or tissue, which can regenerate much easier and quicker than physiologically old tissue such as nodes and internodes. Murthy and Pyati (2001) also reported that the physiological age of an explant is an important factor in regeneration. With the second method, regenerants or clusters of them were excised just next to the node. New protocorms

then arose from the basal part of the incised regenerants or clusters of them and not from the nodes. The first method proved to be more appropriate when many generations of regenerants at different developmental phases are desired from one regenerant in a short period of time. With the second procedure, plant segments regenerated only once (except for media E and F) and regenerants could be subcultured only in larger or smaller clusters, which could be removed from the base without destroying some of the smaller ones only with difficulty. Larger regenerants inside a single cluster can also obstruct the further regeneration and growth of smaller, not fully developed structures or protocorms to regenerants.

Media A, B and C are less appropriate in terms of root formation because the roots developed more slowly and in significantly smaller numbers than with medium D (Fig. 2a-2c). Moreover, most of the regenerants on media A, B and C showed no root formation and for that purpose needed to be subcultured onto medium D just after regeneration. Most of the vegetative regenerants were already ready for acclimatization after 90 days of subcultivation on medium D. The habitus of regenerants on medium D was similar to adult *Phalaenopsis* plants (Fig. 2d). Medium D proved to be a successful subcultivation medium for regenerants formed on media A, B and C.

4 CONCLUSIONS

In summary, our results showed that the average time needed for regeneration of plantlets with well developed leaves on culture media A, B, C and D was shortened by two to three weeks in comparison with data in the reviewed literature (Arditti and Ernst, 1993; Tisserat and Jones, 1999). Moreover, medium D gave rise to plantlets ready for acclimatization after only 90 days. When a large number of regenerants are desired, we recommend medium A (60 days for a large number of clusters) and then subcultivation to medium D (another 60 days), a total of 120 days to produce vigorous plantlets for transplanting to pots, which is a two-month shorter time period than with Park et al. (2002) (six months). Generative regenerants only arose directly from dormant buds, never from later developed protocorms. None of the culture media tested proved to be appropriate for mass generative regenerant production. The multiplication factor of generative regenerants on medium C was only 0.54, which is significantly the best among all the media used. We managed to obtain direct regeneration without undesirable callus formation on successful culture media A, B, C and D and thus to shorten the time needed for formation of first regenerants and reducing the possibility of the occurrence of somaclonal variability. Two ways of excising vegetative regenerants from the nodes (just next to the node or with 3 – 5 mm of the basal part of the regenerated dormant bud on the node preserved) were of major influence on the later development of new protocorms and regenerants.

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