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In Vitro Propagation of *Chlorophytum borivilianum* Using Encapsulated Shoot Buds

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Summary

In vitro propagation of rare Indian medicinal herb 'Safed Musli' (*Chlorophytum borivilianum*) was achieved using encapsulated shoot buds. Four millimetre long shoot buds encapsulated in 3.0 % sodium alginate matrix polymerised by 100 mM solution of $CaCl_2.2H_20$ yielded best results. Storage conditions, gel matrix media and period in storage influenced the *in vitro* regrowth potential of shoot buds when transferred on standard multiplication (SM) medium containing MS salts and 5.0 mg l^{-1} BAP. Encapsulated shoot buds stored on wet agar-gel and kept under culture room conditions of light and temperature (45 µmol m^{-2} s^{-1} and 28±2 °C, respectively) showed more than 80 % sprouting within 3 weeks. Dark stored (4 °C) encapsulated shoot buds on agar-gelled wet medium showed more than 90 % sprouting after 7 d of storage. The per cent response in terms of regrowth potential declined to 60 % when buds were stored for more than 30 d attaining a low of 20 % in another 30 days. Supplementing alginate matrix with sucrose and MS salts (AL_{MSS}) produced better results as compared to that with MS salts alone (AL_{MS}) or without any of them (AL_W). All the sprouted shoot buds, irrespective of their storage conditions, produced normal shoots on SM and multiplied at a rate of 2.5-fold per subculture of 21 d each. More than 70 % microshoots could be rooted on the medium containing $^3/_4$ MS salts and 2.0 mg l^{-1} IBA. Rooted plantlets were hardened successfully and grown under greenhouse conditions where they produced normal tuberous roots. This method of micropropagation opens possibilities for storage of shoot buds during off-season of production and facilitate transport of germplasm with ease.

Zusammenfassung

***In Vitro* Vermehrung von *Chlorophytum borivilianum* mittels eingekapselter Triebknospen.** Durch die Verwendung von eingekapselten Triebknospen gelang es, die seltene indische Medizinalpflanze 'Safed Musli' (*Chlorophytum borivilianum*) *in vitro* zu vermehren. Vier Millimeter lange in durch eine 100 mM $CaCl_2.2H_20$-Lösung polymerisierte 3.0 % Natriumalginatmatrix eingekapselte Triebknospen erzielten die besten Resultate. Die Lagerbedingungen, das Gelmatrixmedium und die Lagerdauer beeinflussten das Austriebspotential der Triebknospen *in vitro*, wenn diese auf Standardvermehrungsmedium (SM) mit MS-Salzen und 5.0 mg l^{-1} BAP gesetzt wurden. Eingekapselte Triebknospen, die in feuchtem Agargel unter Kulturraumbedingungen hinsichtlich Licht und Temperatur (45 µmol m^{-2} s^{-1} bzw. 28±2 °C) gelagert wurden, zeigten in den ersten drei Wochen zu über 80 % Sprossbildung. Eine 7-tägige Lagerung bei 4 °C und Dunkelheit führte bei über 90 % in feuchtem Agargelmedium eingekapselten Triebknospen zur Sprossbildung. Bei einer Knospenlagerdauer über 30 Tage verringerte sich das Austriebspotential auf unter 60 % und erreichte nach weiteren 30 Tagen einen Tiefstand von 20 %. Der Zusatz einer Alginatmatrix in Kombination mit Saccharose und MS-Salzen (AL_{MSS}) führte zu besseren Ergebnissen als die Zugabe einer Alginatmatrix nur mit MS-Salzen (AL_{MS}) oder reiner Alginatmatrix (AL_W). Alle sproßbildenden Triebknospen bildeten unabhängig von den Lagerbedingungen auf SM normale Sprosse aus, die sich in Subkulturen innerhalb von 21 Tagen um das 2,5-fache vermehrten. Die Bewurzelungsrate betrug auf Medium mit $^3/_4$ MS-Salzen und 2.0 mg l^{-1} IBA mehr als 70 %. Bewurzelte Pflänzchen konnten erfolgreiche abgehärtet werden und bildeten unter Gewächshausbedingungen die gewohnten knollenartigen Wurzeln aus. Die hier beschriebene Methode der Mikrovermehrung eröffnet Möglichkeiten, Triebknospen außerhalb der Produktionsphase zu lagern und erleichtert außerdem den Transport von Erbplasma (Genmaterial).

Key words. Germplasm conservation – low temperature storage – micropropagation – rare Indian medicinal herb – Safed Musli

Introduction

Chlorophytum borivilianum (Liliaceae), commonly known as 'Safed Musli', is an endangered medicinal herb (NAYAR and SASTRY 1988) valued for the dried fasciculated storage roots. These are reputed to have aphrodisiac properties and form an important ingredient of herbal tonics prescribed in Ayurvedic system of medicine in India (KIRTIKAR and BASU 1975). Due to its high economic value, efforts have been devoted to use tissue culture biotechnology for conservation and commercial cultivation of this medicinal herb (PUROHIT et al. 1994; SURI et al. 1999; DAVE et al. 2003). Recently, this laboratory has taken-up its large-scale production using micropropagation technology for supply of propagules. *C. borivilianum* has a very short growing season spread over 90–100 d (July–October) and therefore the *in vitro* production should match with the planting season. To be able to make the planting material available for field transfer, the production schedule commences from February and is completed by the end of August. September to January (5 months) is off-season for production of *C. borivilianum* in culture and therefore the shoots are simply multiplied on fresh media every three weeks. In view of saving space, time and resources during this period slow growth, cryopreservation, encapsulation and cold storage of shoot buds are considered as promising options.

Natural unipolar propagules like microbulbs, rhizomes, protocorms (STANDARDI and PICCIONI 1998; DATTA et al. 1999; SAIPRASAD and POLISETTY 2003), nodal cuttings (DANSO and FORD-LLOYD 2003) and shoot buds (UOZUMI and KOBAYASHI 1995; MICHELI et al. 2002) besides bipolar somatic embryos (GRAY and PUROHIT 1991; PATEL et al. 2002) have been subjected to encapsulation for storage, easy handling, transport, delivery and their establishment under *in vitro* and/or *extra vitrum* conditions.

Although a variety of natural and synthetic polymers are available for encapsulation sodium alginate is the most commonly used gel-matrix (GANAPATHI et al. 1994), because of its easy gelling properties, non-toxicity and low cost. Different concentrations of sodium alginate ranging from 1.5 % to 6.0 % have been used for different systems (BAPAT et al. 1987; AHUJA et al. 1989; VIJ et al. 1991; SAKAMOTO et al. 1995; DATTA et al. 1999; MONDAL et al. 2002). It has been suggested that this difference in concentration may be due to quality of alginate, which differs from brand to brand (NAYAK et al. 1998) and batch to batch (AHUJA et al. 1989).

The present investigation was aimed to develop methods for encapsulation of young shoot buds of *C. borivilianum*, which could be stored during off-season of production and subsequently used as propagules for initiation of the production cycle. They can also be transported easily especially for exchange of *in vitro* germplasm.

Materials and Methods

Shoot cultures maintained on standard multiplication (SM) medium (PUROHIT et al. 1994) containing basic MS salts (MURASHIGE and SKOOG 1962) and 5.0 mg l^{-1} BAP served the source of young shoot buds. Shoot buds of varying lengths (3.0, 4.0 and 5.0 mm) were dissected out aseptically from the cluster of proliferating shoots under the stereomicroscope. Isolated buds were blot dried on a filter paper and were transferred into varying concentrations (1, 3 and 5 %) of sodium alginate dissolved in double distilled water (AL_w); liquid MS (AL_{MS}) and also MS salts supplemented with 3 % sucrose (AL_{MSS}). Drops of sodium alginate solution containing shoot buds each were then slowly dropped into 100 mM concentration of $CaCl_2.2H_2O$ solution and stirred continuously up to 40 min on a magnetic stirrer. Calcium alginate beads were formed as a result of the ion exchange between Na^+ and Ca^{+2} ions. Thereafter, the calcium chloride solution was decanted and properly encapsulated beads washed in autoclaved double distilled water three times and finally transferred in a glass vessel for storage. The different concentrations of sodium alginate were tested in order to optimize the size, shape and texture of alginate beads for bud sprouting. Finding that the best beads were formed only with 3 % alginate, this concentration was used in all the experiments. Shoot buds encapsulated in different gel-matrix-media were transferred in a glass vessel containing 50 ml of 0.8 % water agar and kept either in culture room (CR) conditions of light at 28±2 °C or in dark at 4 °C in a cold room (DR). To avoid stickiness, some of the beads were transferred to a bottle containing autoclaved talcum powder and were also stored at two different conditions (CR and DR) as described above.

Comparative proliferation potential of the unencapsulated and encapsulated shoot buds (ESB_S) were evaluated with respect to:

1. per cent response in terms of shoot bud sprouting both under CR and DR conditions;
2. longevity and survival of shoot buds under CR and DR conditions;
3. potential of shoot buds for further multiplication and growth.

Proliferation efficiency of the encapsulated shoot buds (ESB_S) stored for different time period was tested. ESB_S stored both in CR and DR conditions (for 7, 15, 21, 30, 45 and 60 d) were transferred to SM medium. The effect was evaluated with respect to:

1. days taken for shoot bud proliferation;
2. per cent response of alginate beads with respect to bud sprouting;
3. number of shoots formed per shoot bud during subculture of 21 d for 63 d.

Shoots (3.0–3.5 cm) derived from unencapsulated and encapsulated shoot buds were separated and transferred on 3/4th strength MS medium containing 2.0 mg l^{-1} IBA, standard rooting medium (SR) for rooting (PUROHIT et al. 1994). Rooted plantlets were hardened as described by DAVE et al. (2003). Hardened plants were transferred in polybags containing a mixture comprising sand and farmyard manure (1:1) and kept under greenhouse conditions. The data were recorded on per cent rooting, growth and per cent survival of plantlets.

The entire operation of selection, isolation, encapsulation, complexation and transfer of beads in storage vessels was carried out under aseptic conditions on a laminar flow clean air bench. All the media, encapsulation matrices and complexation solution and others

wherever required were autoclaved at 1.06 kg cm^{-2} pressure for 17 min. pH of the MS medium prepared for gel matrix or shoot multiplication was adjusted to 5.8±0.02. All the cultures for their growth were kept under 16 h photoperiod (45 µmol m^{-2} s^{-1} cool white light provided by Philips fluorescent tubes) in a growth room at 28±2 °C and a relative humidity of 50–60 %.

Design of experiments and statistical analyses

Six ESB_S or shoot buds were inoculated in each vessel and three replicates were taken for each treatment. Experiments were repeated three times. To compare the three factors i.e. CR/DR conditions, gel matrices (AL_{MSS}, ALM_S and AL_W) and duration of storage, three factorial CRD was used whereas to compare gel matrices and duration of storage under DR condition, two factorial CRD was used. The data were analysed statistically and conclusions drawn.

Results

Encapsulated shoot buds (ESB_S) having smooth texture appropriate for the sprouting were produced when 3.0 % sodium alginate was used. The beads formed on lower percentage (1.0 %) of sodium alginate were weak and leaky even after 40 min in complexation medium. The beads produced with 5.0 % sodium alginate were very hard and prevented shoot bud proliferation on SM medium. Size of the shoot buds greatly influenced the encapsulation process and formation of beads. Though the shoot buds measuring 3.0 mm or less were good to encapsulate such shoot buds did not proliferate on the medium and turned yellow. The best response, however, was obtained when approximately 4.0 mm size shoot buds were taken for encapsulation. Such shoot buds proved to be most viable propagules and were easy to handle. With 5.0 mm size shoot buds, the beads were very large and irregular in shape. Such shoot buds also showed poor proliferation. ESB_S formed in different combinations of alginate matrices kept in CR or DR conditions showed varied response.

Alginate beads stored on dry substrate (talc) under both CR and DR conditions showed poor proliferation potential. Such shoot buds died within two weeks of their inoculation on the SM medium. The deterioration of shoot buds was rapid in those kept under CR conditions as compared to those kept in DR conditions. No further experiments were conducted with such beads.

Unencapsulated shoot buds stored under light conditions showed proliferation in more than 90 % cases, which multiplied further during subsequent subcultures on SM medium. Under DR conditions the shoot buds showed senescence within 7 d and there was no further growth. ESB_S stored on wet substrate (0.8 % water-agar) proved to be better under both the conditions of light and temperature. Those shoot buds, which were transferred to SM medium and kept under CR conditions after their encapsulation, started proliferation piercing through the gel matrix after 4–5 d of their inoculation. More than 80 % ESB_S showed proliferation and produced 4–5 shoots within 3 weeks. Such shoots multiplied further at a rate of 2.5-fold during every sub-culture of 21 d each.

Shoot buds encapsulated in different gel-matrix-media stored under CR and DR conditions for different durations were tested for their potential of regrowth on SM medium. The data pooled for all the factors in a trifactorial experiment revealed insignificant interaction. However, the data for storage conditions when analysed individually showed significantly higher percentage of proliferation in the shoot buds stored under DR conditions as compared to CR conditions . Similarly the response of gel-matrix was nearly uniform in buds stored for 7 d under DR conditions. This potential declined significantly in next 7 d. The best response, however, was obtained on AL_{MSS} (Table 1). The ESB_S stored for 7 d in DR conditions showed 92 % sprouting. The emergence and elongation of shoots took place in about 8 d in culture, which grew further and multiplied on SM medium. The response in terms of regrowth, however, varied with the type of gel-matrix-media used for encapsulation. Shoot buds encapsulated only with the sodium alginate in double distilled water (AL_W) showed poor response as compared to those, which contained MS salts alone (AL_{MS}) or MS salts together with sucrose (AL_{MSS}). The per cent response showed a declining trend more rapidly in those buds, which were encapsulated in AL_{MS} gel-matrix. Longest duration of survival and best per cent response was obtained in shoot buds encapsulated in AL_{MSS} gel-matrix. The shoot buds showed more than 60 % proliferation up to 30 d in dark storage (Table 2). The bud sprouting efficiency declined when ESB_S were stored for more than 30 d attaining a low of 20 % after 60 d.

Table 1. Effect of storage period and gel-matrix-media on regrowth potential of encapsulated shoot buds of *C. borivilianum* on SM medium containing MS salts and 5.0 mg l^{-1} BAP

Duration of storage	Gel matrices			
	AL_{MSS}	AL_{MS}	AL_W	Mean
07 d	87.50^{α}	89.33^{α}	89.33^{α}	88.72^{x}
15 d	$86.67^{\alpha,\beta}$	80.16^{β}	70.83^{γ}	79.22^{y}
	87.08^{a}	$84.75^{a,b}$	80.08^{b}	83.97

CD at 5 % for gel matrices= 5.04
CD at 5 % for number of days= 4.11
CD at 5 % for interaction gel matrices x number of days= 7.13
Note : Respective means having different letters differ significantly (P≤0.5) using ANOVA

Table 2. Effect of gel-matrix-media and duration of storage under DR conditions on regrowth potential of encapsulated shoot buds of *C. borivilianum* on SM medium containing MS salts and 5.0 mg l^{-1} BAP

Duration of storage	Gel matrices			
	AL_{MSS}	AL_{MS}	AL_{W}	Mean
07 d	92.33[a]	92.33[a]	92.33[a]	92.33[u]
15 d	92.33[a]	83.00[b]	71.66[b,c]	82.33[v]
21 d	83.00[b]	71.67[b,c]	66.70[c]	71.78[w]
30 d	61.00[c]	40.67[d,e]	32.67[e]	44.77[x]
45 d	45.66[d]	21.66[f]	11.00[g]	26.11[y]
60 d	21.66[f]	11.00[g]	11.00[g]	14.56[z]
	66.00[α]	53.39[β]	46.56[γ]	55.32

CD at 5 % for gel matrices= 3.78
CD at 5 % for number of days= 5.35
CD at 5 % for interaction gel matrices x number of days= 9.26
Note : Respective means having different letters differ significantly (P≤0.5) using ANOVA

Once the normal growth was achieved in both the unencapsulated or encapsulated shoot buds on SM medium, the shoots multiplied normally without any morphological aberrations. Roots were initiated in 3.0–3.5 mm long micro-shoots derived from encapsulated and unencapsulated shoot buds. No differences in rooting efficiency were observed in micro-shoots obtained from either encapsulated or unencapsulated buds and the rooting behavior and survival were similar to our earlier observations (PUROHIT et al. 1994). More than 70 % rooting was achieved in shoots inoculated on SR medium.

Rooted plantlets were successfully hardened with more than 90 % success. Such plants transferred in polybags and kept in greenhouse during growing season grew vigorously and produced normal tuberous roots (Fig. 1).

Discussion

The present paper reports the possibility of encapsulation of shoot buds of *C. borivilianum* and their storage during off-season. Since encapsulation saves space, time, energy and resources, it demonstrates advantages over conventional method of shoot multiplication by repeated subculturing. The encapsulated propagules of *C. borivilianum* have remained viable for the period as long as 30 d as has been reported in *Camellia sinensis* (MONDAL et al. 2002), mulberry (MACHII 1992), *Betula* (LEE et al. 1990) and cassava (DANSO and FORD-LLOYD 2003). A solution of 3 % sodium alginate and 100 mM $CaCl_2{:}2H_20$ yielded good quality beads with shoot buds. In case of *Camellia sinensis*, storage of artificial seeds at 4 °C reduced the time period for axillary bud proliferation but in the present case the shoot bud proliferation was delayed by 4–5 d under similar conditions of storage.

In the present investigation, we have optimized use of 3 % sodium alginate for encapsulation of shoot buds. Storage of beads on wet substrate like water-agar proved to be better under both CR and DR conditions. There was no dehydration and desiccation of beads till 60 d though the response declined considerably beyond 30 d. Use of talc adversely affected the storability and regrowth potential of encapsulated shoot buds in present investigation. VIJ et al. (2001) have, however, found coating of synseeds with talc to be beneficial in *Dendrobium* protocorm encapsulation.

The present studies opened up the possibility for an alternate method of maintaining shoot cultures during off-season. The storage period of 30 d achieved during present investigation could be extended by improving gel-matrix-media and other conditions. This has also made transport of germplasm convenient.

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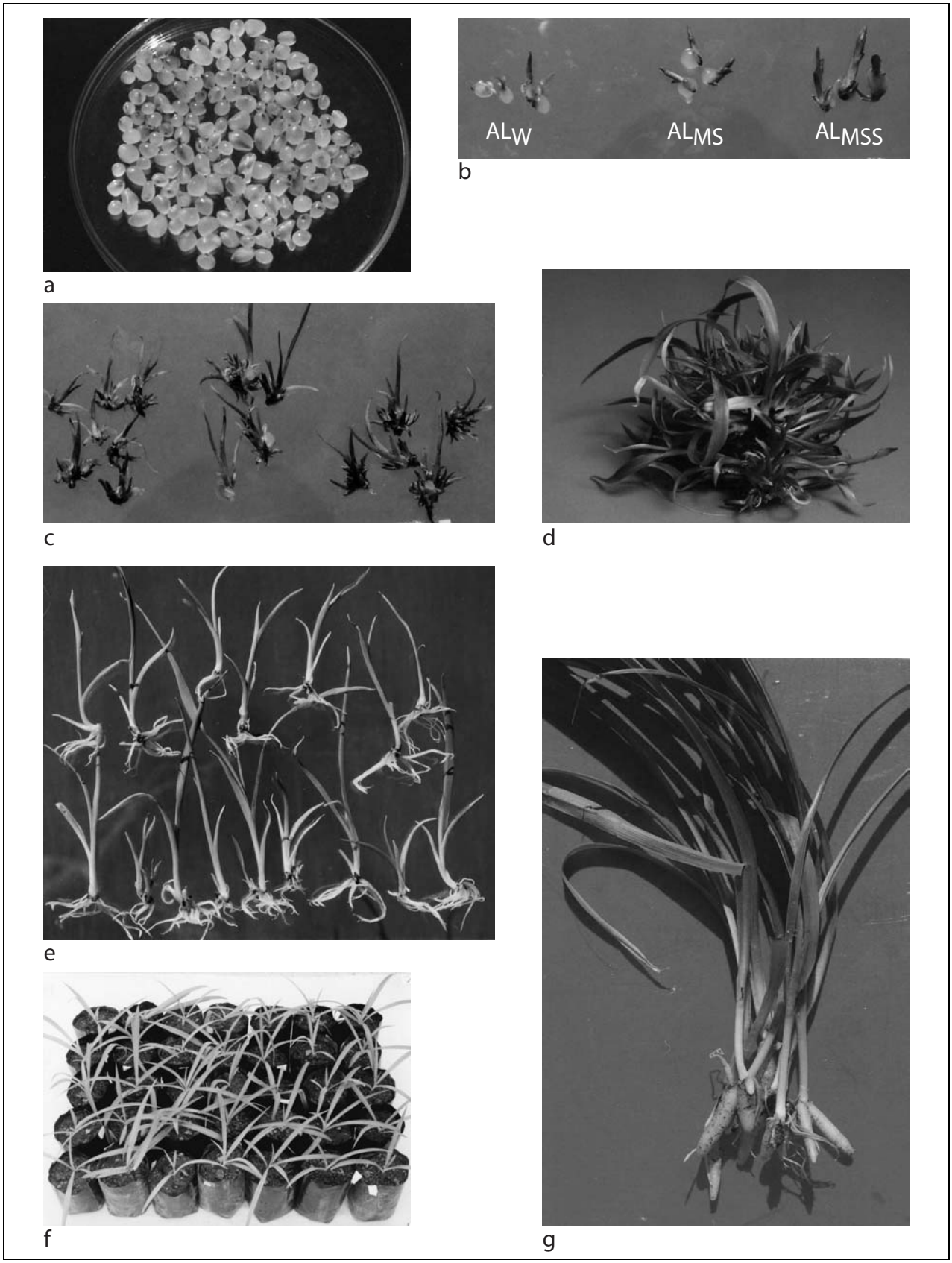


Fig. 1. *In vitro* propagation of *C. borivilianum* using encapsulated young shoot buds (a–g). (a) Encapsulated shoot buds (ESB_s); (b) Sprouting of ESB_s on SM medium after 30 days of storage in dark at 4 °C; (c) Sprouted ESB_s after 21 days of culture on SM medium; (d) Cluster of shoots after 63 days of culture on SM medium; (e) Rooted plantlets on SR medium; (f) Hardened plants in polybags; (g) Development of normal fasciculated roots after one month of growth in polybags.

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