Production of virus-free orchid
*Cymbidium aloifolium* (L.) Sw.
by various tissue culture techniques

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Abstract

Orchids are affected by many viruses resulting in poor growth, yield and quality, and an overall decline in population. Cymbidium mosaic virus (CymMV) is one of the common orchid viruses found in *Cymbidium* species but it infects different orchid genera. In this study *Cymbidium aloifolium* was propagated *in vitro* using MS medium at different strength (1.0, ½, and ¼) with or without 0.5 mg/l BAP (6-benzylaminopurine) and 0.5 mg/l NAA (Naphthalene acetic acid). To provide disease-free planting material, source plant for *in vitro* propagation needs to be screened for pathogenic viruses. In the present study, *in vivo*-grown source (mother) plants and tissue culture-derived plants of *C. aloifolium* were tested for CymMV virus using Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). All the tissue cultured plants were found to be 100% virus-free whereas the *in vivo* grown source plants were highly affected by CymMV virus (83.33%). The virus-free *in vitro* plantlets were multiplied in large scale and then acclimatized on earthen pot containing a mixture of cocopeat, litter and clay in the ratio of 3:2:1. Eighty five percent of acclimatized plantlets survived making this method an efficient mass production system for high quality virus-free *C. aloifolium* for commercial floriculture and germplasm preservation.

Keywords: Biological sciences, Plant biology
1. Introduction

Plant viruses and virus-like diseases damage almost all plants causing huge economic loss, through reduction in quality and quantity of products. The exact data for crop loss by virus are not available for Nepal and the occurrence and severity of viral diseases differ with season, location and crop variety. Orchid is a major floricultural crop worldwide. At present, orchids are valued for cut flowers, potted plant and herbal medicine (Pant and Raskoti, 2013). As in other plants, in orchids pathogenic viruses tend to accumulate within the host and spread to healthy plants reducing yield and quality (Torres et al., 2000). Virus-infected orchids show stunted growth, produce fewer and poor quality flowers. Currently appropriate quarantine measure, eradication of infected stocks and provision of virus-free planting stocks remain the main strategy to control viral diseases in orchids. Plant tissue culture has been widely used to produce virus-free plants of orchids (Taşkı̈n et al., 2013).

Orchids are mostly affected by two viruses; Cymbidium mosaic virus (CymMV) and Odontoglossum ring spot virus (ORSV) (Khentry et al., 2006; Liu et al., 2013; Wong et al., 1994; Zettler et al., 1990). CymMV was first described by Jensen (1951) who found black necrotic spotting on Cymbidium spp. and named as Cymbidium Black Streak virus. CymMV virus is responsible for flower colour breaking and necrosis. It causes a mosaic of irregularly shaped chlorotic or necrotic lesions to appear on infected plants with sunken patches on leaves (Hu et al., 1993). Besides Cymbidium, CymMV infects a number of other orchids and non-orchid plants. Certain infected plants may be symptomless but are still a source of infection for other neighbouring plants. A rod-shaped Odontoglossum ringspot virus (ORSV) was observed in Odontoglossum grande (Jensen and Gold, 1951). These viruses were later detected in Cymbidium where it was called Cymbidium diamond mottle and in Cattleya, where it causes flower variegation and ring spots on leaves (Jensen, 1970). Both viruses are highly infectious, stable and usually present in orchid juice in high concentrations and reduce the plant growth (Pearson and Cole, 1986). These viruses are mostly spread through contaminated tools, pots and manual contact. Destruction of infected plant is the only way to prevent the spread of disease. These viruses are reported in a wide variety of orchid genera like Cymbidium, Dendrobium, Oncidium, Phalaenopsis, Vanilla etc. (Chien et al., 2015; Khentry et al., 2006; Liu et al., 2013; Zeng et al., 2009; Zhang et al., 2005).

The detection of virus in plant is a critical requirement for successful commercial production of orchids. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is one of the most popular techniques for the detection of plant viruses. This method includes conjugated antibodies, antigen antibodies and substrate for test reaction. DAS-ELISA uses antiviral antibodies to trap the viruses from plant samples; it also detects the bound viruses (Hu et al., 1993).
Cymbidium aloifolium (L.) Sw., an endangered epiphytic orchid has great horticultural value in Nepal. Habitat loss, indiscriminate collection for illegal trade and increasing demand are depleting the natural population of this orchid (Pradhan et al., 2013). This situation is aggravated by the spread of viral diseases. In vitro culture techniques such as shoot tip culture, meristem culture, micrografting, cryo-therapy etc. have been extensively used for elimination of pathogens from infected stocks to produce disease-free plants (Idowu et al., 2009). Hence, development and rapid mass production of disease-free planting material protocol is an urgent need. Keeping this in view, we established an in vitro system to produce the virus-free plants of Cymbidium aloifolium by using tissue culture techniques.

2. Materials and methods

2.1. Plant materials

Immature capsule of Cymbidium aloifolium obtained from natural habitat (wild source plants) from Hetauda, Nepal was selected as explants for in vitro culture. Randomly selected young fresh leaves (around 4 cm length) derived from wild source plants and tissue cultured plants were used for screening the CymMV virus.

2.2. Sterilization of explants

Immature capsule was washed thoroughly under running tap water and soaked in detergent water containing Tween-20 (0.05% v/v) for half an hour. Then, the capsule was again washed under running water until all the detergent goes off. These capsules were surface sterilized in sterile laminar flow hood by immersing the capsule in sodium hypochlorite solution (1%) for 5 min followed by 70% ethanol for 2 min. Finally the treated capsules were rinsed with sterile water thrice and dried on sterile filter paper.

2.3. Establishment of culture media and culture conditions

Murashige and Skoog (MS, 1962) medium was used as a basal medium for present investigation. Different strength of MS media i.e. full (1.0), half (1/2), quarter (1/4) and full strength (1.0) of MS medium supplemented with 0.5 mg/l BAP (6-benzylaminopurine) and 0.5 mg/l NAA (Naphthalene acetic acid) were used for in vitro culture. All the media were adjusted to pH 5.8 with 0.1 N NaOH or HCl before autoclaving and solidified with 0.8% w/v Difco Bacto Agar. About 20 ml medium was dispensed into each sterile culture tubes which were sealed with aluminium foil and autoclaved at 120 °C and 15 psi for 20 min.
2.4. Inoculation of seeds and explants preparation

The surface sterilized capsule was opened longitudinally with the help of sterile scalpel and seeds were scooped out and used as explants for culture initiation. The cluster of extracted seeds was spread over different tested media i.e. 1.0 MS, ½ MS, ¼ MS and 1.0 MS + 0.5 mg/l BAP + 0.5 mg/l NAA. The cultures were grown in culture room and incubated at 25 ± 2 °C under a 16/8 h light/dark cycle. Light was provided by cool white fluorescent lamps (1000 lux) (Philips, India).

After few weeks, seeds were differentiated into protocorms and finally gave rise to whole plantlets. Twenty one days old protocorms were used to produce artificial seeds by encapsulating with 2%, 3% and 4% sodium alginate and 0.2 M calcium dihydrate solution. Single protocorms, young shoot tip as well as artificial seeds were sub-cultured on 1.0 MS, ½ MS, ¼ MS and 1.0 MS + 0.5 mg/l BAP + 0.5 mg/l NAA. Young fresh leaves obtained from plantlets derived from seeds, protocorms, shoot tip and artificial seeds were used for detection of CymMV virus. The percentage of seed germination or viability of all explants was calculated as below:

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\% \text{ of Seed germination/Viability} = \frac{\text{No. of seed swelling of embryo}}{\text{No. of total seeds used for study}} \times 100
\]

2.5. Detection of CymMV by DAS-ELISA

Double antibody sandwich enzyme linked immunosorbent (DAS-ELISA) assay was used for detection of *Cymbidium* mosaic virus (CymMV) of in vitro and in vivo explants using antibodies of CymMV (Agdia, USA). DAS-ELISA was performed by following the protocol described by Clark and Adams (1977). According to this protocol, microtitre plate wells (96 wells) were coated with 200 μl of antibody of CymMV (dilute 1:200, Agdia, USA) diluted in coating buffer at 1:1000 ratio. The plate was incubated at 37 °C for 4 h. Following incubation, about 200 mg of fresh young leaves containing midrib and vein were taken for plant extraction. Plant extracts were prepared by grinding leaf tissue in sterile mortar and pestle and homogenized in 5 ml of maceration buffer containing phosphate buffered saline (PBS) with 0.5 ml/l Tween 20 and 2% polyvinylpyrrolidone (PVP). The homogenized sap were then centrifuged at 2000 rpm for 3 min and the supernatant was loaded into the ELISA plate at the rate of 200 μl per well. The plate was then incubated at 37 °C for 4 h. After incubation, the conjugate antibody (antibody linked with enzyme alkaline phosphatase; dilute 1:200; Agdia, USA) was mixed with the buffer at 1:1000 ratios and 200 μl of the mixture was added to each well. The plate was again incubated at 37 °C for 3 h. Plates were thoroughly washed with 0.01 M phosphate buffered saline (pH 7.4) at each stage after incubation for at least three times to remove the traces of soluble reactants that could cause non-specific reactions. The final step of DAS-ELISA is to add substrate which is p-nitro phenyl phosphate (pNPP, Agdia, USA). The substrate solution is freshly prepared in substrate buffer at the rate of 1 mg/ml and dispensed.
into the plate at the rate of 200 µl per well. These plates were incubated for 30–60 minutes for colour reaction. Strong yellow colour was observed in the positive control and CymMV positive samples. Finally, the optical density (OD) of the reaction mixture was recorded at 405 nm wavelength with the help of ELISA plate reader for quantitative result. The colour reaction was then stopped by adding 5 µl of 5% NaOH solution in each well. This experiment was performed in triplicates.

Samples were considered positive when OD values were more than 1.0. In present study, in vitro regenerated plantlets of Cymbidium species which was free from CymMV in repetitive virus test by DAS-ELISA experiments were used as (−)ve control and lyophilized powder of Cymbidium species (obtained directly from Agdia, USA) which showed presence of CymMV was used as (+)ve control. The percentage of infection of CymMV of all explants was calculated as below:

\[
\% \text{ of Infection (CymMV)} = \frac{\text{No. of infected plants}}{\text{No. of total plants used for study}} \times 100
\]

2.6. Hardening of in vitro plantlets

In vitro plantlets (4–5 cm long) with strong roots were carefully taken out from culture bottles and gently washed under running tap water to remove the traces of media. They were then treated with 1% fungicide (Bavistine) solution for 5 min and transplanted to earthen pot containing cocopeat, litter and clay in the ratio of 3:2:1 with the sphagnum moss topping. Potted plants were covered with perforated transparent polythene bag for 3–4 weeks and were kept in shade to avoid direct exposure of sunlight. The potted plants were watered daily and fertilized weekly with nitrogen, phosphorous and potassium (NPK 20:10:10). After one month, the plantlets were transferred to greenhouse for their normal growth. Observations on the development of seedlings were recorded every week.

3. Results

Young fresh leaves of in vivo and in vitro regenerated plantlets of Cymbidium aloifolium were used as explants for screening of CymMV virus using DAS-ELISA technique. Plantlets regenerated from in vitro culture of seeds, protocorms, shoot tips and artificial seeds of C. aloifolium were tested for CymMV virus and compared with wild mother plant. In the present study, CymMV virus was found only on mother plant whereas all other in vitro explants were free from virus.

In vitro culture of seeds on different tested media swelled considerably within 7 to 15 weeks of culture (Table 1) and became greenish and rounded structure called protocorm (Fig. 1a). Later, protocorms were differentiated to give rise to healthy plantlets within 27 weeks of culture on 1.0 MS + 0.5 mg/l BAP + 0.5 mg/l NAA. On this condition, about 98.33% of seeds were successfully germinated. This condition was followed by full strength of MS medium (1.0 MS) which showed
### Table 1. In-vitro germination and regeneration of plantlets from different explants of *Cymbidium aloifolium*.

| Explants          | Medium          | Initiation of Germination/Development | Development of seedling (Avg. weeks) | % of Seed Germination/viability/Development |
|-------------------|-----------------|--------------------------------------|-------------------------------------|---------------------------------------------|
| Seed              | 1.0 MS          | 10                                   | 30                                  | 93.33                                       |
|                   | ½ MS            | 12                                   | –                                   | 87.5                                        |
|                   | ¼ MS            | 15                                   | –                                   | 82.5                                        |
|                   | 1.0 MS + 0.5BAP + 0.5NAA | 7                                  | 27                                  | 98.33                                       |
| Protocorm         | 1.0 MS          | 5                                    | 20                                  | 91.66                                       |
|                   | ½ MS            | 6                                    | 22                                  | 83.33                                       |
|                   | ¼ MS            | 6                                    | 23                                  | 66.67                                       |
|                   | 1.0 MS + 0.5BAP + 0.5NAA | 6                                  | 23                                  | 66.67                                       |
| Shoot tip         | 1.0 MS          | 4                                    | 19                                  | 83.33                                       |
|                   | ½ MS            | 5                                    | 20                                  | 83.33                                       |
|                   | ¼ MS            | 7                                    | 20                                  | 66.67                                       |
|                   | 1.0 MS + 0.5BAP + 0.5NAA | 3                                  | 18                                  | 91.66                                       |
| Artificial seed (2%) | 1.0 MS          | 5                                    | 20                                  | 100                                         |
|                   | ½ MS            | 5                                    | 22                                  | 66.67                                       |
|                   | ¼ MS            | 4                                    | 20                                  | 83.33                                       |
|                   | 1.0 MS + 0.5BAP + 0.5NAA | 5                                  | 22                                  | 100                                         |
| Artificial seed (3%) | 1.0 MS          | 5                                    | 19                                  | 100                                         |
|                   | ½ MS            | 6                                    | 22                                  | 83.33                                       |
|                   | ¼ MS            | 6                                    | 23                                  | 83.33                                       |
|                   | 1.0 MS + 0.5BAP + 0.5NAA | 7                                  | 22                                  | 75                                          |
| Artificial seed (4%) | 1.0 MS          | 7                                    | 21                                  | 100                                         |
|                   | ½ MS            | 8                                    | 22                                  | 83.33                                       |
|                   | ¼ MS            | 8                                    | 24                                  | 66.67                                       |
|                   | 1.0 MS + 0.5BAP + 0.5NAA | 8                                  | 23                                  | 75                                          |

Culture conditions: – MS medium, 25 ± 2 °C, 32 weeks, 16/8 h. photoperiod, 6 replicates were used in each condition.
93.33% of seed germination and took 30 weeks of culture for complete seedling development (Pradhan et al., 2013). However, ½ MS and ¼ MS media gave only protocorm and their further development process was retarded after 32 weeks of culture. Protocorms and shoot tips were obtained from in vitro cultured seeds and were used as explants for further experiment. Artificial seeds were produced by encapsulating protocorm with 2%, 3% and 4% of sodium alginate solution and 0.2 M CaCl₂ solution (Fig. 1b). Each artificial seed, shoot tip and protocorm were sub-cultured on different strength of MS media i.e. 1.0 MS, ½ MS, ¼ MS and 1.0 MS + 0.5 mg/l BAP + 0.5 mg/l NAA under aseptic condition in laminar air flow chamber. All the tested conditions responded variedly for germination and development of protocorm, shoot tip and artificial seeds.

In present study, individual protocorm was started to develop within 5–6 weeks of culture in all tested medium. It was found that 1.0 MS medium was found to be effective for earlier development of complete seedling (20 weeks of culture) and also showed highest percentage i.e. 91.66% of protocorm development than other tested conditions (Table 1). Similarly, shoot tip explants responded within 3 to 7 weeks of culture. The complete seedling was obtained within 18 weeks of culture on 1.0 MS + 0.5 mg/l BAP + 0.5 mg/l NAA (Fig. 1c) and showed 91.66% of development. In vitro culture of 2% alginate coated artificial seeds started to germinate within 4 to 5 weeks of culture on all tested conditions and obtained
100% seed germination on 1.0 MS and 1.0 MS + 0.5 mg/l BAP + 0.5 mg/l NAA medium. However, the earlier response of seedling development from 2% alginate coated was observed on 18 weeks of culture on 1.0 MS + 0.5 mg/l BAP + 0.5 mg/l NAA. Similarly, in vitro germination of 3% and 4% alginate coated artificial seeds took 5 to 6 weeks of culture and 7 to 8 weeks of culture respectively. Both 3% and 4% artificial seeds showed 100% viability on 1.0 MS medium and took 19 weeks of culture and 22 weeks of culture for fastest development of seedling respectively in comparison to other tested conditions (Table 1). Large number of multiple shoots was produced after 24 weeks of culture from protocorms (Fig. 1d), shoot tips and artificial seeds. Young in vitro plantlets derived from seeds, protocorms, shoot tips and artificial seeds were tested for CymMV using DAS-ELISA and compared with mother plant and (+)ve control (obtained directly from Agdia, USA). It was found that all the in vitro plantlets were 100% free from CymMV virus whereas mother plant showed 83.33% of virus infection (Table 2).

### 4. Discussion

In present study, green immature capsule of *Cymbidium aloifolium* was taken for in vitro culture. The germination of orchid seeds in nature is a unique phenomenon. Orchid seeds are very small and do not contain any food reserve to feed the growing embryo. They require mycorrhizal fungus for germination which supplies the embryo with sugar and nutrients (Arditti et al., 1981). Hence, plant tissue culture technique was found to be effective means for the germination and propagation of orchid’s asymbiotically. In present study, 1.0 MS medium

### Table 2. Detection of *Cymbidium* mosaic virus (CymMV) in wild (mother plant) and in vitro explants of *Cymbidium aloifolium*.

| Explants               | Antigen | Absorbance (405 nm) mean ± SD | % of Infection (CymMV) | Inference           |
|------------------------|---------|--------------------------------|------------------------|---------------------|
| Control (+) ve         | CymMV   | 1.70 ± 0.59                    |                        | Severely Positive   |
| Control (−) ve         | "       | 0.23 ± 0.04                    |                        | Negative            |
| Mother plant           | "       | 1.09 ± 0.23                    | 83.33                  | Severely Positive   |
| Seed                   | "       | 0.18 ± 0.05                    | 0                      | Negative            |
| Protocorm              | "       | 0.16 ± 0.07                    | 0                      | Negative            |
| Shoot tips             | "       | 0.19 ± 0.06                    | 0                      | Negative            |
| Artificial seed (2%)   | "       | 0.18 ± 0.05                    | 0                      | Negative            |
| Artificial seed (3%)   | "       | 0.17 ± 0.05                    | 0                      | Negative            |
| Artificial seed (4%)   | "       | 0.17 ± 0.05                    | 0                      | Negative            |

6 replicates were used for each plant sample, CymMV- *Cymbidium* mosaic virus, Virus index: < 0.3 = negative; 0.3–0.99 = mild positive; >1.0 = severely positive.
supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA was found to be effective condition for *in vitro* germination and seedling development from seeds as well as from shoot tips and 2% artificial seeds whereas hormone free 1.0 MS medium was found to be favourable for earlier development of seedling from protocorm, 3% artificial seeds and 4% artificial seeds. This was identified on the basis of time taken for seed germination and their further differentiation into seedling. MS medium is highly enriched with macro and micro elements with different vitamins (Hossain et al., 2009). Due to this, highest percentage of seed germination and seedling development was found on MS medium alone and MS medium supplemented with BAP and NAA compared to ½ and ¼ strength of MS medium as they have low amount of micro and macro elements. Similar findings were also reported in *Cymbidium aloifolium* (Pradhan et al., 2014), *Cymbidium iridioides* (Pongener and Deb, 2010), *Coelogyne suaveolens* (Deb, 2008) and *Malaxis khasiana* (Deb and Temjensangba, 2006).

It has been reported that orchids are affected by at least 25 different orchid viruses around the world (Liu et al., 2013). Of these, CymMV virus is one of the most prevalent and economically important followed by *Odontoglossum* ringspot virus (ORSV) (Khentry et al., 2006; Wong et al., 1994; Zettler et al., 1990). CymMV is stable and widespread infection can occur, once the virus is introduced into a nursery. So, it is desirable to test the plant materials for the presence of viruses before being used for mass propagation by tissue culture technique. Serological (ELISA), biological indexing and molecular methods (RT PCR) are generally used for detection and identification of plant viruses (Taşkınpasa et al., 2013). DAS-ELISA technique has been used by different researchers because of its simplicity, accuracy and cost effective (Chien et al., 2015; Hu et al., 1993; Khan et al., 2003; Milošević et al., 2012). In the present study also, DAS-ELISA technique was found to be the effective technique for accurate detection of CymMV virus of *in vivo* and *in vitro* plantlets of *Cymbidium aloifolium*.

CymMV virus was not found in all the *in vitro* regenerated plantlets used in this study which might be due to the absence of causal agent of CymMV. However, the source mother plant was highly infected with CymMV (Fig. 2). This result showed that CymMV was not seed transmitted which was supported by the findings reported by Khentry et al. (2006). Hence the use of seed propagated plantlets as explants should be one of the most prominent approaches to establish the virus free orchid plants and high quality germplasm.

When the plants were infected with virus, chemical therapy will not be effective to control it. Meristem culture technique is the most common method for obtaining virus-free plants. Different researchers have also reported that meristem culture is effective for obtaining virus-free plants of different crops (Cybularz-Urban and Hanus-Fajerska, 2006; Fidan et al., 2009; Kumar et al., 2009; Milošević et al., 2012).
However, extracting the shoot meristem and regenerating them into plants is difficult, time consuming and requires crop-specific expertise mostly in orchids. Therefore seed, protocorm, shoot tip and artificial seed culture could be used as an alternative to produce virus-free plants of orchids. Virus-free orchids show faster and healthier growth and produce larger inflorescences (Chia and He, 1999; Liu et al., 2013). The virus-free in vitro plantlets were multiplied (Fig. 1e) and transferred to the earthen pots in greenhouse for acclimatization (Fig. 1f). Eighty five percent of them survived and grown to maturity. Hence, the results obtained from the present study are very important to develop a protocol for virus-free plants by using tissue culture techniques which increases the better quality yield for local orchid growers as well as for germplasm preservation.

5. Conclusion

Tissue culture techniques have been employed for large-scale of clonal propagation of orchids. Also, the international trade of virus-free orchids is currently increasing. Therefore, it is very important to screen all the plants used for in vitro propagation in order to prevent the spread of viruses. The present study established a protocol for regeneration of virus-free Cymbidium aloifolium plants from infected mother stock.
Declarations

Author contribution statement

Shreeti Pradhan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tripti Regmi: Contributed reagents, materials, analysis tools or data.

Mukunda Ranjit: Analyzed and interpreted the data.

Bijaya Pant: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

Arditti, J., Michaud, J.D., Oliva, A.P., 1981. Seed germination of North American orchids. I. native California and related species of Calypso, Epipactis, Goodyera, Piperia, and Platanthera. Bot. Gaz., 442–453.

Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475–483.

Chia, T.F., He, J., 1999. Photosynthetic capacity in Oncidium (Orchidaceae) plants after virus eradication. Environ. Exp. Bot. 42, 11–16.
Chien, K.W., Agrawal, D.C., Tsay, H.S., Chang, C.A., 2015. Elimination of mixed ‘Odontoglossum ringspot’ and ‘Cymbidium mosaic’ viruses from Phalaenopsis hybrid ‘V3’ through shoot-tip culture and protocorm-like body selection. Crop Prot. 67, 1–6.

Cybularz-Urban, T., Hanus-Fajerska, E., 2006. Therapeutic effect of cytokinin sequence application on virus-infected Cattleya tissue cultures. Acta Biol. Cracoviensia Ser. Bot. 48, 27–32.

Deb, C.R., 2008. Effects of different factors on immature embryo culture, PLBs differentiation and rapid mass multiplication of Coelogyne suaveolens (Lindl.) Hook. Int. J. Exp. Bio. 46 (4), 243.

Deb, C.R., Temjensangba, S., 2006. In vitro propagation of threatened terrestrial orchid, Malaxis khasiana Soland ex: Swartz through immature seed culture. Int. J. Exp. Bio. 44 (9), 762.

Fidan, H., Baloglu, S., Koc, G., Birisk, N., 2009. New virus diseases for Turkey detected in onion and garlic: onion yellow dwarf virus and shallot latent virus. Proceedings of the third plant protection congress of Turkey, 15–18.

Idowu, P.E., Ibitoye, D.O., Ademoyegun, O.T., 2009. Tissue culture as a plant production technique for horticultural crops. Afr. J. Biotechnol. 8, 3782–3788.

Hossain, M.M., Sharma, M., Pathak, P., 2009. Cost effective protocol for in vitro mass propagation of Cymbidium aloifolium (L.) Sw. – a medicinally important orchid. Eng. Life Sci. 6, 444–453.

Hu, J.S., Ferreira, S., Wang, M., Xu, M.Q., 1993. Detection of cymbidium mosaic virus, odontoglossum ringspot virus, tomato spotted wilt virus, and potyviruses infecting orchids in Hawaii. Plant Dis. 77, 464–468.

Jensen, D.D., 1951. Mosaic or black streak disease of Cymbidium orchids. Phytopathology 41, 401–414.

Jensen, D.D., Gold, A.H., 1951. A virus ringspot of Odontoglossum orchid: symptoms, transmission, and electron microscopy. Phytopathology 41, 648–653.

Jensen, D.D., 1970. Virus diseases of orchids in the Netherlands. Neth. J. Plant Pathol. 76, 135–139.

Khan, M.S., Hoque, M.I., Sarker, R.H., Muehlbach, H.P., 2003. Detection of important plant viruses in invitro regenerated potato plants by double antibody sandwich method of ELISA. Plant Tissue Cult. 13, 21–29.

Khentry, Y., Paradornuwat, A., Tantiwiwat, S., Phansiri, S., Thaveechai, N., 2006. Incidence of Cymbidium Mosaic Virus and Odontoglossum Ringspot Virus on In
Vitro Thai Native Orchid Seedlings and Cultivated Orchid Mericlones. Kasetsart J. (Nat Sci.) 40, 49–57.

Kumar, S., Khan, M.S., Raj, S.K., Sharma, A.K., 2009. Elimination of mixed infection of Cucumber mosaic and Tomato aspermy virus from Chrysanthemum morifolium Ramaty cv. Pooja by shoot meristem culture. Sci. Hortic. 119, 108–112.

Liu, F., Han, Y., Li, W., Shi, X., Xu, W., Lin, M., 2013. Incidence of Cymbidium mosaic virus and Odontoglossum ringspot virus affecting Oncidium orchids in Hainan Island, China. Crop Prot. 54, 176–180.

Milosevic, S., Subotic, A., Bulajic, A., Djekic, I., Jevremovic, S., Vucurovic, A., Krstic, B., 2011. Elimination of TSWV from Impatiens hawkerii Bull: and regeneration of virus-free plant. Elect. J. Biotechnol. 14, 3–4.

Milošević, S., Cingel, A., Jevremović, S., Stanković, I., Bulajić, A., Krstić, B., Subotić, A., 2012. Virus elimination from ornamental plants using in vitro culture techniques. Pestic. Fitomed. 27, 203–211.

Pant, B., Raskoti, B.B., 2013. Medicinal orchids of Nepal. Himalayan Map House Pvt Ltd., Nepal.

Pearson, M.N., Cole, J.S., 1986. The effects of Cymbidium mosaic virus and Odontoglossum ringspot virus on the growth of Cymbidium orchids. J. Phytopathology. 117, 193–197.

Pongener, A., Deb, C.R., 2010. Asymbiotic culture of immature embryos, mass multiplication of Cymbidium iridioides D. Don. and the role of different factors. Int. J. Pharma. Bio. Sci. 1 (1), 1–14.

Pradhan, S., Regmi, T., Parmar, G., Pant, B., 2013. Effect of different media on in vitro seed germination and seedling development of Cymbidium aloifolium (L.) Sw. Nepal J. Sci. Technol. 14, 51–56.

Pradhan, S., Tiruwa, B., Subedee, B.R., Pant, B., 2014. In vitro germination and propagation of a threatened medicinal orchid: Cymbidium aloifolium (L.) Sw. through artificial seed. Asian Pac. J. Trop. Biomed. 4 (12), 971–976.

Taşkin, H., Baktemur, G., Kurul, M., Büyükalaca, S., 2013. Use of tissue culture techniques for producing virus-free plant in garlic and their identification through real-time PCR. Scientific World J.

Torres, A.C., Fajardo, T.V., Dusi, A.N., Resende, R.D.O., Buso, J.A., 2000. Shoot tip culture and thermotherapy for recovering virus-free plants of garlic. Hortic. Bras. 18, 192–195.
Wong, S.M., Chng, C.G., Lee, Y.H., Tan, K., Zettler, F.W., 1994. Incidence of
cymbidium mosaic and odontoglossum ringspot viruses and their significance in
orchid cultivation in Singapore. Crop Prot. 13, 235–239.

Zeng, Y.J., Mo, R., Liu, Z.X., Wang, J.H., 2009. A survey to and pathogen
identification of virus diseases of orchid in Hainan. Chinese J. Trop. Agric. 29,
17–19.

Zettler, F.W., Ko, N.J., Wisler, G.C., Elliott, M.S., Wong, S.M., 1990. Viruses of
orchids and their control. Plant Dis. 74, 621–626.

Zhang, Y.J., Li, G.F., Shi, Z.W., 2005. Pathogeny identification on virus disease of
Vanilla planifolia in Hainan Province. J. Northwest Sci-Tech Univ. Agric For. 33
(Suppl), 135–136.