

# *In Vitro* Propagation of Endangered Orchid, *Vanda pumila* Hook.f. through Protocorms Culture

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## Abstract

*The Vanda pumila* is a monopodial orchid with beautiful flowers that are native to Thailand but now found across South Asia. The immature seeds of *Vanda pumila* were used for *in vitro* culture and then the protocorms developed were used as explants for seedling development and mass propagation. Protocorms were cultured on 1/2 MS (Murashige and Skoog, 1962) medium fortified separately with Kinetin (Kn), 6-Benzyl amino purine (BAP) and Gibberellic Acid (GA3) each in different concentrations as (0.5 mg/L, 1.0 mg/L and 2.0 mg/L) well as each on each concentrations of each medium supplemented with 5% and 10% coconut water (CW) respectively. The greatest number of shoots ( $9.50 \pm 0.29$  shoots per culture) was developed on 1/2 MS medium fortified with 1.0 mg/L Kn plus 10% CW and the longest shoots ( $0.78 \pm 0.07$  cm per culture) developed on 1/2 MS medium fortified with 2.0 mg/L BAP plus 10% CW. The shoots derived from protocorms were then developed on 1/2 MS medium fortified with three different rooting hormones viz. Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and  $\alpha$ -Naphthalene acetic acid (NAA), each in four concentrations (0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L) as well as 1.0 mg/L of each hormone supplemented with 10% CW. The 1/2 MS medium fortified with 0.5 mg/L IAA was found to be the most effective condition for the development of maximum number of root ( $5 \pm 0.0$  roots per culture) and root length ( $0.93 \pm 0.07$  cm). Hence, the present study could be useful for standardizing the protocol for mass propagation of the endangered orchid *V. pumila*.

## Keywords

Culture, Micropropagation, MS Medium, Kinetin, Rooting

## 1. Introduction

Orchids are an immensely beautiful group of plants with extraordinary variety. The colour of the flowers of these members of Orchidaceae family is undoubtedly their greatest charm. As few orchid flowers are pollinated in nature, the few seed pods are formed and those seeds that are produced are unlikely to germinate [1]. In nature, orchid seeds require specific mycorrhizal fungal associations to germinate, so less than 1% do that [2]. *In vitro* culture in contrast, fungal associations are not required for germination, as first demonstrated by Knudson in 1922 [3] [4].

Six species of *Vanda*, including *Vanda pumila* have been recorded in Nepal [5] and its synonym is *Trudelia pumila*. *V. pumila*, which is closely related to *V. cristata*, is commonly known as “The Dwarf Vanda”. This orchid species is also found in North East India, Nepal, Myanmar, China, Laos, Thailand and Vietnam at the elevation of 1200 - 2300 m [5]. *V. pumila* is important for breeding cultivars [6] and most of the species have great medicinal value. The root of *Vanda tessellata*, for example is traditionally used for an antidote for scorpion stings and a remedy for bronchitis and rheumatism. A paste of its leaves is used to treat fever, cough, piles and other ailments [7] [8] [9]. In addition, the leaves of *Vanda teres* and *Vanda cristata* are used to treat fever [10]. *Vanda cristata* also has anti-cancer property [11].

Because of the restricted population size and expected medicinal properties of *V. pumila*, it is required to develop an effective conservation strategy to save this plant for future. Plant tissue culture technique provides an opportunity to preserve and commercialize the number of rare and threatened orchid species [12]. Hence, the present study was carried out to standardize the protocol for mass propagation and *ex-situ* conservation of this species.

## 2. Materials and Methods

### 2.1. Plant Material and Protocorm Culture

Immature capsules of *Vanda pumila* were collected from the garden of the Central Department of Botany, Kirtipur, Kathmandu. The capsules were surface sterilized with 1 - 2 drops of TWEEN 20 and washed with running tap water for at least half an hour. The capsules were then dipped in 1% sodium hypochlorite solution for 15 minutes before they held with forceps and submerged in 70% ethanol where they were rapidly set a flame for a few seconds. The capsules were then rinsed three times in sterile water. These surface sterilized capsules were then cut longitudinally with a sterile scalpel and the exposed seeds were removed with forceps and transferred to an agar gel (0.8% per liter) nutrient medium for seed germination. Different strength of MS [13] medium (full strength, 1/2 and 1/4<sup>th</sup> strength), were used to germinate the seeds and then the protocorms developed from the seeds were used for shoot proliferation.

The eight month old protocorms that developed on the hormone free MS medium (average of five each protocorms about 0.3 mm to 0.4 mm diameter) were

sub cultured on 1/2 strength MS medium supplemented respectively with BAP (0.5 mg/L, 1.0 mg/L, and 2.0 mg/L), Kinetin (0.5 mg/L, 1.0 mg/L, and 2.0 mg/L) and GA3 (0.5 mg/L, 1.0 mg/L and 2.0 mg/L). Each concentration of each hormone was tested both alone and supplemented with both 5% CW and 10% CW. The sequential growth and proliferation of *Vanda pumila* was then evaluated. The pH of the medium was maintained at a slightly acidic 5.7 - 5.8 by adding 0.1 N NaOH and 0.1N HCl before agar was added. The mediums were autoclaved at 15 psi and 121°C for 20 minutes. Cultures were incubated at 24°C ± 1°C provided with 1000 lux illumination by cool white fluorescent light of 16 hours light and 8 hours dark regime. Each culture medium had 4 replicates.

## 2.2. Rooting of Shoots

After 12 weeks of *in vitro* growth, protocorm's shoots measuring 0.8 cm - 1 cm developed on 1/2 MS medium fortified with different concentrations (0.5 mg/L, 1.0 mg/L, and 2.0 mg/L) of IAA, IBA and NAA alone as well as on a 1.0 mg/L concentrations of each hormone supplemented with 10% CW.

## 2.3. Statistical Analysis

Average value of week was taken for *in vitro* seed germination. Data of shooting and rooting were presented as mean and standard error. Data were analyzed by one way ANOVA with F-statistics at 95% confidence interval using SPSS version 20.

## 3. Results and Discussion

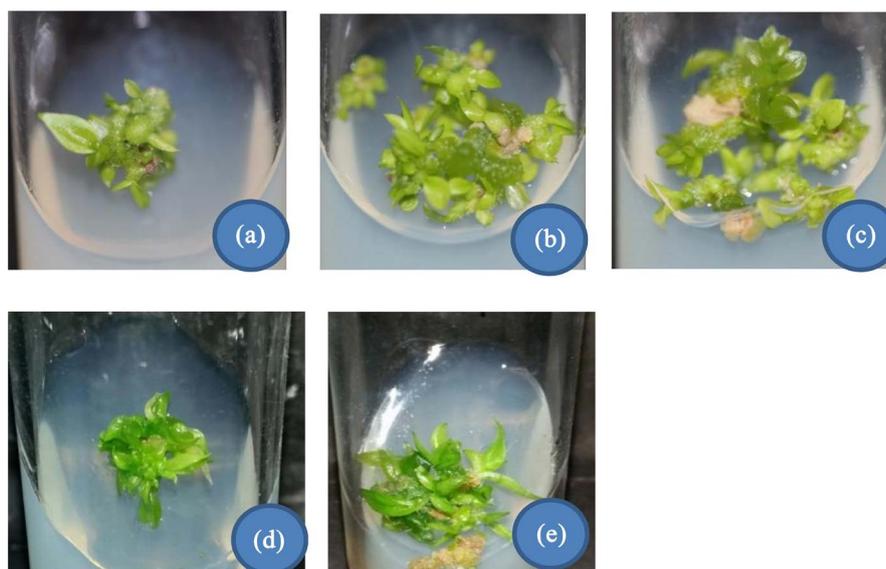
### 3.1. Shoot Development through Protocorm Culture

To develop the shoots of *Vanda pumila*, the eight month old protocorms measuring 3 mm - 4 mm in diameter derived from the *in vitro* seed germination of *V. pumila* were sub-cultured on 1/2 MS medium supplemented with different concentrations 0.5 mg/L, 1.0 mg/L and 2.0 mg/L of BAP, Kn and GA3 on their own as well as on concentration of each hormone supplemented with both 5% and 10% CW. Within the four weeks of culture the protocorms in all the tested conditions had responded, undergoing multiplication and exhibiting leaf primordia. The most effective medium for shoot multiplication was 1/2 MS medium supplemented with 1.0 mg/L Kn and 10% CW ( $9.50 \pm 0.29$  shoots per culture) and the longest shoots were observed on 1/2 MS medium supplemented with 2.0 mg/L BAP and 10% CW ( $0.78 \pm 0.07$  cm per culture). However, 1/2 MS medium supplemented with 1.0 mg/L GA3 and 10% CW yielded the  $2.0 \pm 0.0$  roots per culture with average root length of  $0.42 \pm 0.07$  cm per culture. This finding is encouraging as, according to Teixeira da Silva *et al.* [14], young PLBs are highly meristematic in nature and can be used to improve plant regeneration when they are used as primary explant.

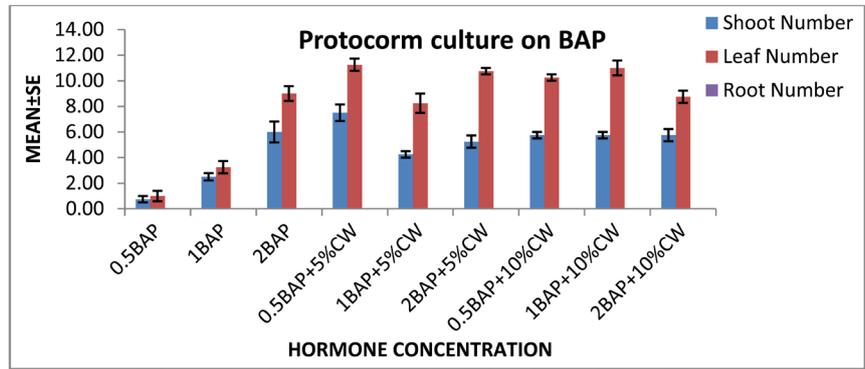
Of the three concentrations of BAP supplemented medium, 1/2 MS medium fortified with 2.0 mg/L BAP yielded the greatest number of shoots (6 shoots per

culture) and the longest shoots ( $0.58 \pm 0.25$  cm) as well as the greatest number of leaves ( $9 \pm 0.57$  leaves per culture) and the longest leaves ( $0.43 \pm 0.48$  cm per culture). This result was similar to the findings of David *et al.* [15] who reported that in case of *Vanda helvola*. They reported that MS medium fortified with either 1.0 mg/L BAP or 2.0 mg/L BAP were the best for protocorms proliferation. Similarly, Luo *et al.* [16] reported that BAP alone was more effective for the proliferation of PLBs of *Dendrobium densiflorum* when combined with NAA and Kn. Regmi *et al.* [17] also reported that MS medium fortified with both 2.0 mg/L BAP and 0.5 mg/L NAA was effective for the protocorms proliferation of *Cymbidium aloifolium*. In the present study, the development of shoot was found to be the highest ( $0.78 \pm 0.07$  cm per culture) on 2 mg/L BAP fortified with 10% CW while the greatest number of shoots ( $7.5 \pm 0.65$  shoots per culture), the greatest number of leaves ( $11.25 \pm 0.48$  leaves per culture) and the longest leaves ( $0.55 \pm 0.29$  cm) were recorded on 0.5 mg/L BAP fortified with 5% CW (Figure 1(c), Figure 2 and Figure 3). However, roots were not developed on any BAP fortified medium either alone or in combination with 5% and 10% CW. Similarly, Gantait and Sinniah [18] reported that 1.0 mg/L BA + 0.5 mg/L IBA fortified with 60 ADS as additives was best for protocorm proliferation on AV-hybrid and that high frequency of PLB conversion was observed in the presence of additives.

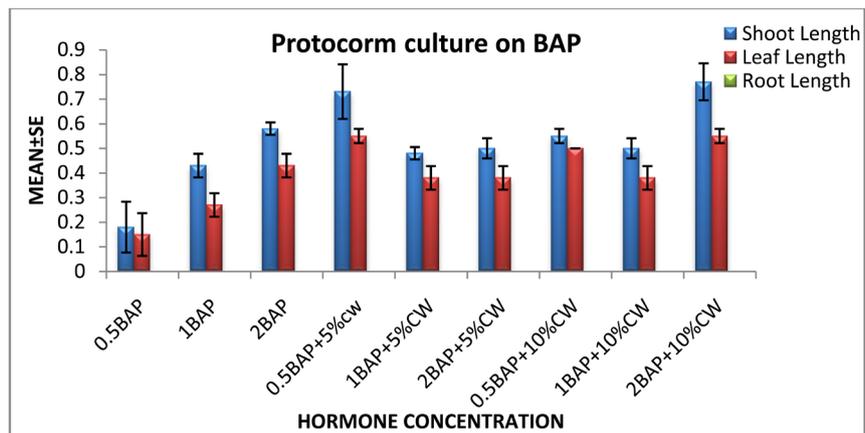
In the case of 1/2 MS medium fortified with different concentrations (0.5 mg/L, 1.0 mg/L, and 2.0 mg/L) of kinetin (Kn), 0.5 mg/L Kn resulted in the earliest response and developed 5 rootless shoots per culture (Figure 4). In contrast, Hrahsal and Thangjam [19] reported that with respect to *Vanda coerulea*, BAP



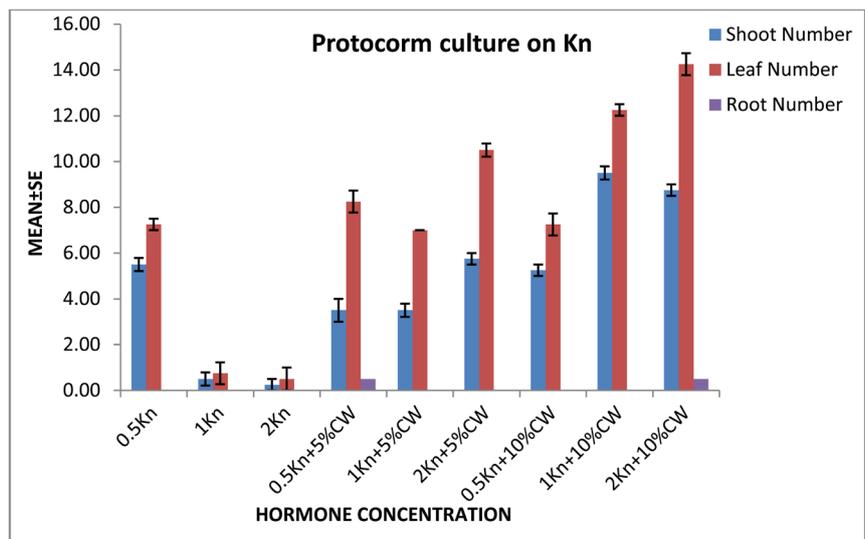
**Figure 1.** *In vitro* shoot development through the protocorms culture of *Vanda pumila* on 1/2 MS medium fortified with different concentration of hormone after 12 weeks of culture. Multiplication of shoots: (a) = 0.5 mg/L Kn, (b) = 1 mg/L Kn + 10% CW, (c) = 0.5 Mg/L BAP + 5% CW, (d) = 1 mg/L BAP + 10% CW and (e) = 0.5 mg/L GA3 + 5% CW.



**Figure 2.** Average numbers of shoots, leaves, and roots produced through the protocorms culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of BAP both alone and in combination with 5% and 10% CW.



**Figure 3.** Average lengths of shoots, leaves, and roots through the protocorms culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of both BAP alone and in combination with 5% and 10% CW.

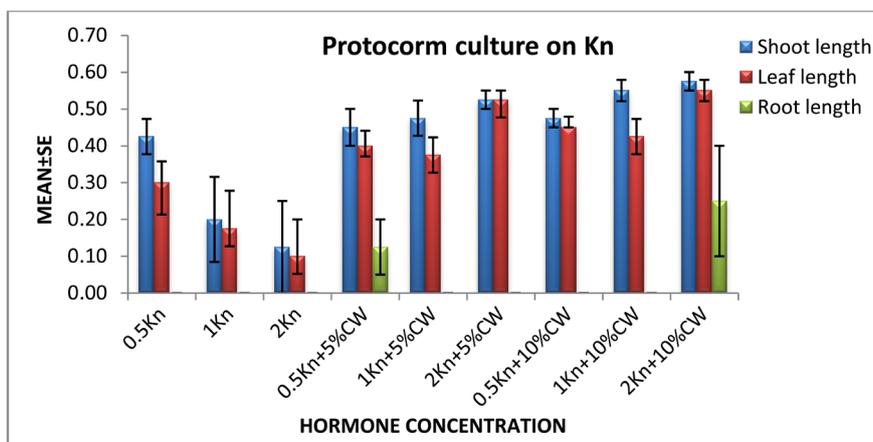


**Figure 4.** Average number of shoots, leaves, and roots through the protocorm culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of Kn with alone and in combination with 5% and 10% CW.

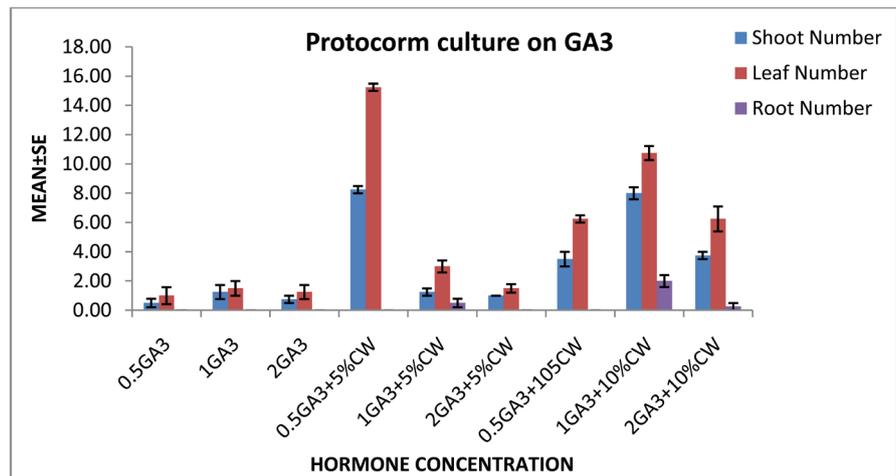
and Kn alone was not effective for protocorm proliferation. In case of 1/2 MS medium supplemented with different concentrations of Kn plus 5% and 10% CW, the greatest proliferation of shoots, was recorded on 1.0 mg/L Kn with 10% CW ( $9.5 \pm 0.29$  shoots per culture). However, the longest shoots ( $0.58 \pm 0.025$  cm per culture) were observed on 2.0 mg/L Kn with 10% CW. This condition resulted in the greatest number of leaves ( $14.25 \pm 0.479$  leaves per culture) and longest leaves ( $0.55 \pm 0.29$  per culture) as well as the greatest number of roots ( $0.5 \pm 0.29$  roots per culture) and longest roots ( $0.25 \pm 0.15$  cm per culture) (Figure 4 and Figure 5). Ng and Saleh [20] reported that 1/2 MS medium fortified with 2.0  $\mu$ M Kn resulted in the highest proliferation of PLBs of *Paphiopedilum*. They also observed that 1/2 MS medium fortified with 20% CW promoted tertiary PLBs proliferation in *Paphiopedilum*. Similarly, Dutta *et al.* [21] reported that, in case of in *Dendrobium aphyllum*, IAA supplemented with Kn was more effective for protocorm proliferation.

In the present study, 1/2 MS medium fortified with different concentrations of GA3 (0.5 mg/L, 1.0 mg/L and 2.0 mg/L) alone resulted in very little multiplication of protocorms as most of the cultures started to become necrotic within five weeks after seedling (Figure 6). Dohling *et al.* [22] reported that seed germination and seedling development in *Dendrobium* species were inhibited when GA3 in medium. Of the 1/2 MS medium supplemented with different concentrations (0.5 mg/L, 1.0 mg/L and 2.0 mg/L) of GA3 plus 5% and 10% CW, the 1/2 MS medium fortified with 0.5 mg/L GA3 plus 5% CW was resulted in the development of the highest number of both shoots ( $8.25 \pm 0.25$  shoots per culture) and leaves ( $15.25 \pm 0.25$  leaves per culture) as well as the longest leaves ( $0.43 \pm 0.25$  cm per culture).

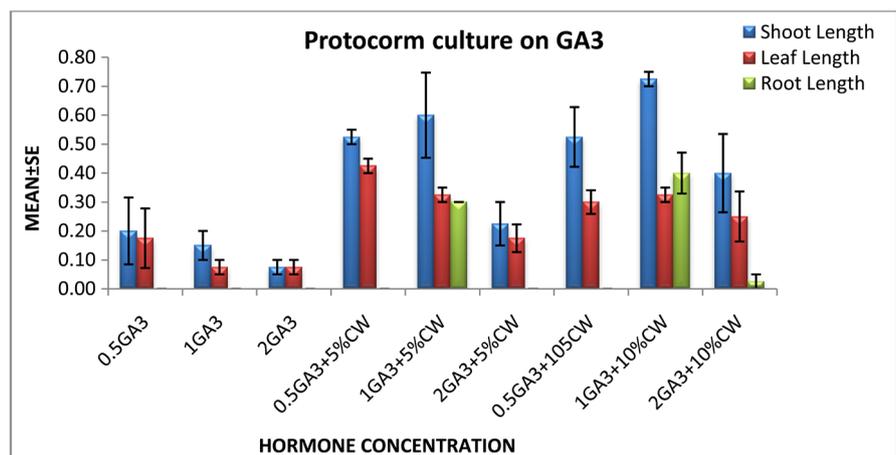
However, the longest shoots ( $0.73 \pm 0.25$  cm per culture), the greatest number of roots ( $2.25 \pm 0.42$  roots per culture) and the longest roots ( $0.40 \pm 0.7$  per culture) were recorded on 1/2 MS medium fortified with 1.0 mg/L GA3 plus 10% CW (Figure 6 and Figure 7).



**Figure 5.** Average length of shoots, leaves, and roots through protocorm culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of Kn alone and in combination with 5% and 10% CW.



**Figure 6.** Average number of shoots, leaves, and root number through protocorm culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of GA3 both alone and in combination with 5% and 10% CW.



**Figure 7.** Average length of shoots, leaves, and roots through protocorm culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of GA3 alone and in combination with 5% and 10% CW.

As Jualang [23] was found the similar report with *Vanda dearie*, all the hormone concentrations supplemented with CW study of *Vanda pumila*, white hair papillae was appeared. Clearly, coconut water plays a vital role in the protocorm proliferation. According to Gnasekaran *et al.* [24], coconut water is added to the tissue culture medium because it contains diphenyl urea, a growth factor which exhibits cytokinin-like responses. The result of this study is partially supported by Saiprasad *et al.* [25], who reported that 0.5 mg/L Kn supplemented with 10% CW was ideal for the protocorm multiplication of *Dendrobium Oncidium* and *Catteleya*, but shoot length was the greatest on 1/2 MS medium supplemented with BAP. The present results were also in accordance with the findings regarding *Dendrobium*, *Oncidium* and *Catteleya* orchid as well as *Paphiopedilum* and *Cymbidium pendulum* [20] [25] [26]. The present study of *V. pumila* revealed that kinetin helped to convert protocorms into shoots and this result was in

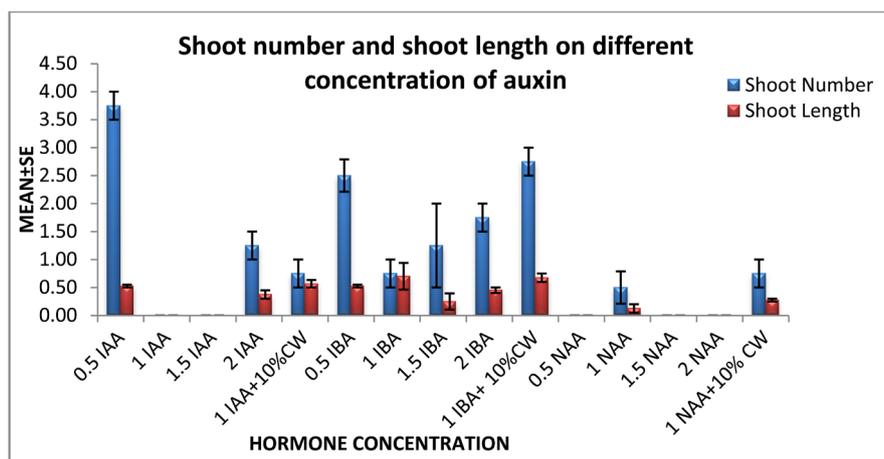
agreement with the findings of *Dendrobium* hybrid [27]. The use of Vacin and Went (VW) medium supplemented with coconut water and 20% tomato extract, produced the greatest proliferation rate for PLBs of Vand Kasem's Delight [28].

### 3.2. Root Development of *Vanda pumila*

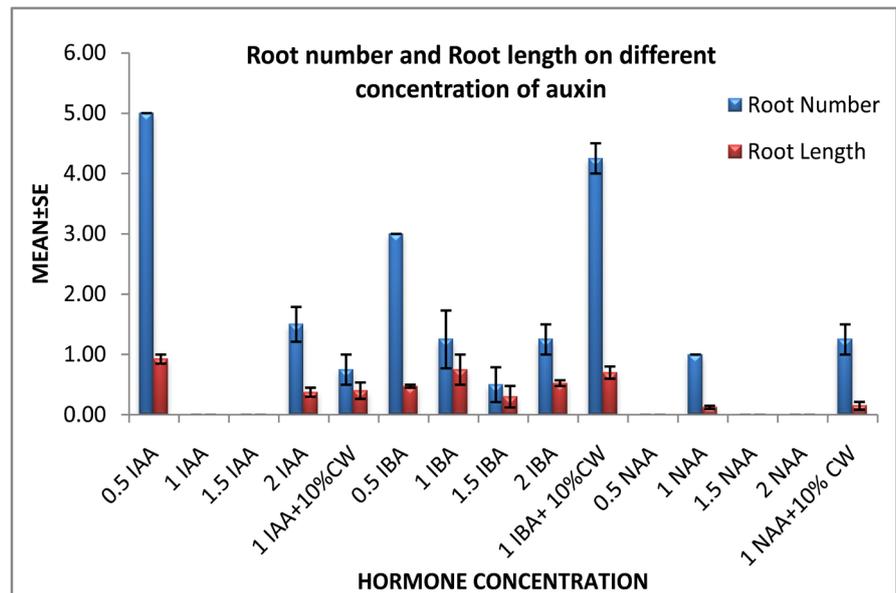
Once they were about 0.5 - 0.8 cm. long, the shoots derived from protocorms grown *in vitro* were cultured on 1/2 MS medium fortified with different concentrations (0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L) of the auxins IAA, IBA and NAA alone as well as on 1.0 mg/L concentrations of each auxin supplemented with 10% coconut water (Figure 8). 1/2 MS medium supplemented with different concentrations (0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L) of IAA alone, the greatest number of roots ( $5 \pm 0.0$  roots per culture) and the longest roots ( $0.93 \pm 0.07$  cm) were found on 1/2 MS medium fortified with 0.5 mg/L IAA (Figure 8 and Figure 9).

Bhattacharjee and Islam [29] reported that, with respect to *Acampe premorsa*, *Agrostophyllum khasianum* and *Phalaenopsis cornorerrri* roots were longer on a full strength MS medium fortified with 0.5 mg/L IAA than on full strength MS medium supplemented with 1.0 mg/L IAA. Hossain *et al.* [30] reported that for *Cymbidium aloifolium*, a medium fortified with 0.5 mg/L IAA promoted the development of stout roots. Similarly, Pant and Thapa [31] found that in case of *Dendrobium primulinum*, MS medium supplemented with 0.5 mg/L IAA resulted in the greatest proliferation of roots. This finding, however, contrasted with that of Rahman *et al.* [32] on *Vanda tessellata*. Bhattacharjee and Islam [33] recorded that *Vanda tessellate* produced the most roots on 1/2 MS medium fortified with 1.0 mg/L IAA and Hrashel and Thangjam [19] reported that 1/2 MS medium fortified with 28.50  $\mu$ M IAA yielded many roots in *Vanda coerulea*.

Regarding 1/2 MS medium supplemented with different concentrations (0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L) of IBA, 0.5 mg/L IBA resulted in the



**Figure 8.** Average number and length of shoots through shoot-tip culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of IAA, IBA and NAA after 12 weeks of culture.

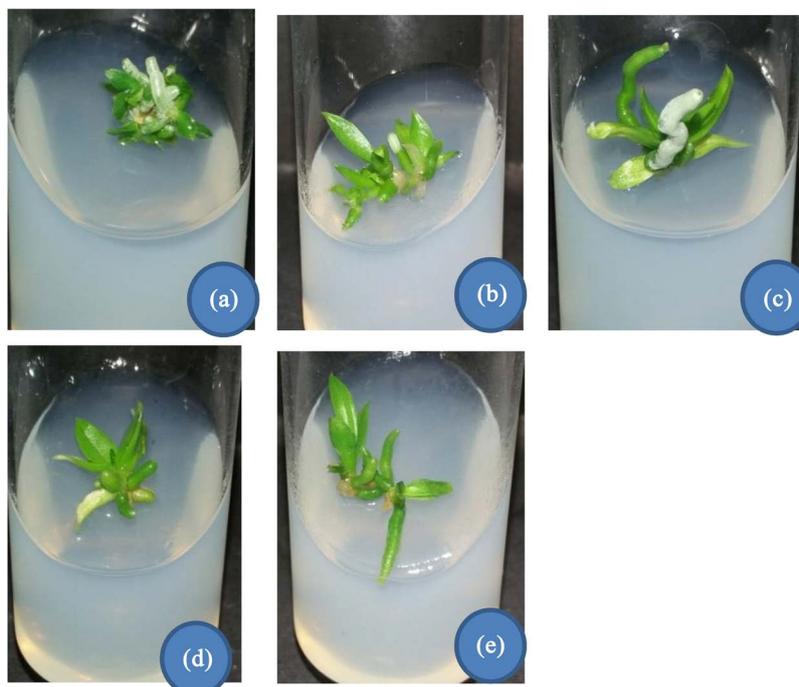


**Figure 9.** Average number and length of roots produced through shoot-tip culture of *Vanda pumila* on 1/2 MS medium supplemented with different concentrations of IAA, IBA and NAA.

development of  $3.0 \pm 0.0$  roots per culture and  $2.5 \pm 0.28$  shoots per culture within 12 weeks of culture. However, the longest roots ( $0.75 \pm 0.25$  cm) and shoots ( $0.70 \pm 0.23$  cm) were found on 1/2 MS medium fortified with 1.0 mg/L IBA. In addition, the roots on this medium were stouter and healthier than they were on mediums made with other concentrations. Rafique *et al.* [34] and Aktar *et al.* [35] found that 1.0 mg/L IBA was maximized both the number and the lengths of roots in the case of *Dendrobium* orchids and Rahman *et al.* [32] found the *Vanda tessellate* also developed good roots on IBA supplemented mediums.

On 1/2 MS medium supplemented with different concentrations (0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L) of NAA, rooting was very poor. In fact, roots developed only on 1.0 mg/L NAA and were very few, ( $1 \pm 0.0$  per culture), as shown in **Figure 8** and **Figure 9**. Rajbahak and Rajkarnikar [36] reported a similar result in the case of *Dendrobium longicornu*. Among the coconut water supplemented mediums, 1.0 mg/L IBA supplemented with 10% CW resulted in the greatest number of roots ( $4.25 \pm 0.25$  roots per culture), longest roots ( $0.70 \pm 0.1$  cm), the most shoots ( $2.75 \pm 0.25$  shoots per culture) and the longest shoots ( $0.68 \pm 0.075$  cm) (**Figure 10(e)**).

Among the tested conditions of Auxin, 1/2 MS medium fortified with 0.5 mg/L IAA was found to maximize both the number and length of the roots of *Vanda pumila*. It was better than other concentrations of IAA, than other rooting hormones (IBA and NAA) and mediums with coconut water. However, in the present study, all the IBA supplemented mediums produced satisfactory number of roots from 0.5 to 3 roots per culture. Similar findings were reported by Pradhan *et al.* [37] who found that IBA was better rooting hormone for *Dendrobium densiflorum* as compare to IAA and NAA.



**Figure 10.** The *in vitro* rooting of *Vanda pumila* on 1/2 MS medium fortified with different concentrations of auxins (IAA, IBA and NAA). (a) = 0.5 mg/L IAA, (b) = 0.5 mg/L IBA, (c) = 1 mg/L IBA, (d) = 1 mg/L NAA and (e) = 1 mg/L IBA + 10% CW after 12 weeks of culture.

#### 4. Conclusion

The present study concluded that among the different tested conditions for shoot development, the 1/2 MS medium fortified with 1.0 mg/L Kn plus 10% CW and 2.0 mg/L BAP plus 10% CW was found to be the more effective for the proliferation of large number of shoots ( $9.50 \pm 0.29$  shoots per culture) and shoot length ( $0.78 \pm 0.07$  cm per culture) respectively from the *in vitro* culture protocorms of *Vanda Pumila*. For rooting, a great number of strong and healthy roots of this species were developed on 1/2 MS medium fortified with 0.5 mg/L IAA ( $5 \pm 0.0$  roots per culture and  $0.93 \pm 0.07$  cm root length per culture). Hence the present result could be useful for the establishment of the standard protocol for the mass propagation and *ex-situ* conservation of this endangered orchid species.

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#### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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