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Nitric oxide promoted rhizome induction in *Cymbidium* shoot buds under magnesium deficiency

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Abstract

Cymbidium shoot buds grown under Mg^{2+} deficiency without naphthalene acetic acid (NAA) showed knotted appearance. Ultrastructure of the cortical cells showed a progressive disorganization and disintegration of chloroplast membranes. The growth of shoots was resumed with the addition of 10 μM NAA. Specific NO scavenger, cPTIO induced deformation in shoot growth in 80 % of cultures. In longitudinal sections of shoots treated with cPTIO, depositions of densely stained particles in cells were observed. These inhibitory responses of cPTIO were ameliorated by 10 μM NAA. The NO donor, sodium nitroprusside (SNP), treated shoot buds displayed rapid senescence followed by necrosis of leaves. Ultrastructure of cortical cells at this stage revealed the endocytosis of mitochondria along with membrane bound cytoplasmic inclusions in the vacuole. A sharp increase in H_2O_2 generation was observed with a little change in the activity of antioxidant glutathione disulfide (GSSG), suggesting NO mediated oxidative stress. Surprisingly, after 4 weeks these necrotic shoots were converted into a globular, embryo like shoot tip with numerous structures termed here as 'neomorph' in its base. Neomorphs were different from protocorm like bodies both anatomically and morphologically. Ultrastructure of the rhizome tip exhibited numerous amyloplast and round mitochondria. At this stage, the generation of high rate of H_2O_2 was masked by GSSG, and the generation of GSSG was proportional with the concentrations of SNP, and not observed in the control (without SNP). The neomorphs were further sub-cultured to medium with different concentrations of SNP or cPTIO. After 4 weeks of culture, only the neomorphs sub-cultured on medium with SNP developed into shoots and approximately ten shoots were observed to emerge from the axils of each rhizome. Ultrastructure of cells of regenerating green neomorphs showed different shapes of mitochondria and chloroplasts and presence of active dictyosomes. The obtained shoots subjected to the acclimatization in polyhouse, expressed good growth with 85 % survival. Therefore it is reasonable to suggest that the process of de-differentiation and re-differentiation leading to rhizome formation under the condition of Mg^{2+} deficiency is NO mediated.

Additional keywords: NAA, neomorph, oxidative stress, rhizome, SNP, ultrastructure.

Introduction

Cymbidium orchids exhibit distinctive ecological diversification, and the basic adaptations of this group of orchids is increased efficiency of mineral use (Roy and Banerjee 2002). At an early stage of their ontogeny, the protocorm like bodies develop into an elongated rhizome which later on differentiates into leaves and pseudobulbs. In nature, increase in number of seedlings through rhizome proliferation and subsequent shoot formation are the way the plants compensate to some extent for the low rate of seed germination (Roy and Banerjee 2002).

Naphthaleneacetic acid (NAA) is very effective in inducing rhizome in *Cymbidium* (Shimasaki and Uemoto 1990). In our previous study with *Cymbidium*, it was observed that rhizome was induced by NAA in absence of macronutrients like N, P, K and Ca, but it failed in absence of Mg (unpublished). Therefore it seems quite likely that Mg^{2+} is required along with NAA to induce rhizome formation in *Cymbidium*. Binding of NAA to plasma membrane is insensitive to monovalent ions, however, it is inhibited by Mg^{2+} or Ca^{2+} above 5 mM concentration (Ray 1977).

Magnesium holds a critical role in activation of some

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Abbreviations: ABA - abscisic acid; cPTIO - 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylinidazoline-1-oxyl-3-oxide; GSSG - glutathione disulfide; MS - Murashige and Skoog; NAA - naphthaleneacetic acid; NPA - N-1-naphthylphthalamic acid; PLBs - protocorm like bodies; ROS - reactive oxygen species; SNP - sodium nitroprusside; TIBA - 2,3,5-triiodobenzoic acid.

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of the important enzymes such as ATPases, ribulose-1,5-bisphosphate (RUBP) carboxylase, RNA polymerase and protein kinases (Cakmak and Kirkby 2008). Mg^{2+} deficiency resulted in dramatic increases in sugar accumulation in leaves (Hermans *et al*. 2004) and impairment of photosynthetic electron transport to CO_2 assimilation (Hermans and Venbruggen 2005).

Nitric oxide is involved in germination and induction of lateral roots. It also modulates the influx of extracellular Ca^{2+} and actin filament organization during cell wall construction in *Pinus bungeana* pollen tubes (Wang *et al*. 2009). Recently, Wang *et al*. (2010b) reported that NO induced programmed cell death (PCD) through mitochondrial pathway which is regulated by Ca^{2+} in tobacco protoplasts. Exogenous application of NO also down-regulated xanthine oxidase mediated generation of $O_2^{\cdot-}$ in *Phalaenopsis* flowers (Tewari *et al*. 2009). Sun *et al*. (2007) reported that sodium nitropruside (SNP), an NO donor, partially reversed iron deficiency induced retardation of plant growth as well as chlorosis, suggesting a link between NO and iron metabolism. Recently, Batasheva *et al*. (2010), reported that export of assimilates from leaves might be regulated NO signaling system. The involvement of NO in plant hormone responses has been well documented. NO mediated cytokinin-induced betalaine accumulation (Scherer and Holk 2000) and ABA-induced stomatal closure (Neill *et al*. 2002). Furthermore, Pagnussat *et al*. (2002) provided evidence about NO and auxin cross-talk during adventitious root formation in cucumber. Later on Correa *et al*. (2004) also reported that NO was able to promote lateral root initiation in roots treated with the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA), and that the promotion of lateral root development by NAA can be prevented by scavenging NO.

The main aim of these experiments was to reply two questions: *1*) is rhizome formation under Mg^{2+} deficiency and in presence of NAA inhibited by NO, and *2*) what role NO plays in absence of NAA and Mg^{2+}.

Materials and methods

Uniform shoot buds (1.5 cm) before leaf initiation of *Cymbidium* hybrid Via del Playa Yvonne (*Cymbidium solana* × *Cymbidium rincon*) was obtained from the culture of inflorescence nodal explants. Borosilicate glass culture tubes with translucent plastic caps (*Magenta*, Chicago, IL, USA), containing 10 cm^3 of modified Murashige and Skoog (MS; 1962) medium were used. Modification was made by withholding $MgSO_4$ and replacing it with equivalent of Na_2SO_4 in order to maintain the supply of sulphur and the osmotic potential. Ultra pure agar (*Invitrogen*, Burlington, Canada) was used. NAA, 2,3,5-tri-iodobenzoic acid (TIBA) and SNP procured from *Sigma* (St. Louis, MO, USA), and 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylinidazoline-1-oxyl-3-oxide (cPTIO) purchased from *Molecular Probes* (Eugene, OR, USA), were cold sterilized with 0.22 µm filter assembly and were mixed with the medium autoclaved at 120 ºC for 15 min. The pH was adjusted to 5.7 with 1 M KOH. The temperature of the culture room was 25 ± 2 ºC, 12-h photoperiod and irradiance of 46 µmol m^{-2} s^{-1} provided by two cool white fluorescent lamps (*Sylvania* daylight *F40 D/RS/SS*). The culture tubes were subjected to bottom cooling as described by Vanderschaeghe and Debergh (1987).

For acclimatization of plantlets, the plantlets obtained from neomorphs (with fully expanded leaves and a height of 5 - 6 cm) and the rhizomes with fully grown rhizome apex and axillary shoots with two to three velamenous roots were retrieved from the culture tubes and washed with tap water and transferred to plastic cups containing autoclaved potting mixture. The mixture comprised of a combination of bark (mango tree), *Perlite* and peat (neutralised with chalk up to pH 6.3) in the ratio 1:1:1 and hydrogels like *Alcosorb-400* (procured from *Alternatives Wholesale Nursery*, Waipu, New Zealand). The plantlets were later transferred to earthenware small pots (8 cm diameter) and were maintained in polyhouse under natural irradiance and the temperature was maintained at 22 ± 2 ºC with the help of locally made desert coolers.

The plantlets were retrieved from their culture vessels and photographs were captured by *Nikon* (Tokyo, Japan) digital camera-*DXM 1200*. For light microscopy, the plant segments were fixed in FAA solution (ethanol + distilled water + formaldehyde + glacial acetic acid, 10:7:2:1, v/v) for 24 h. Segments were included in a paraffin matrix (*Hystoplast*) at 60 ºC and after cooling were cut into 4 µm sections in longitudinal orientation using a rotary microtome. Sections were stained in 1 % safranin and 1 % aniline blue, deparaffined with xylene, slowly rehydrated, and sequentially washed with water and Tris buffered saline (TBS). Sections were then examined by bright field microscopy in a *Nikon Eclipse E 200* microscope and images were captured using *Nikon* digital camera *DXM 1200* attached to the microscope. For transmission electron microscopy, samples were fixed in modified Karnovsky's fluid (David *et al*. 1973) buffered with 0.1 M sodium phosphate buffer at pH 7.4 and at 4 ºC (under vacuum). The tissues were post-fixed in 1 % osmium tetraoxide and dehydrated in graded acetone solutions and embedded in *Araldite CY 212*. Ultrathin sections of 60 - 80 nm were cut using an *Ultracut E* (*Reichert Jung*, Wetzlar, Germany) ultramicrotome and stained in alcoholic uranyl acetate and lead citrate, and examined in a *Philips* transmission electron microscope *EM 300* operated at 80 kV. For scanning electron microscopy, samples were fixed in modified Karnovsky's fluid made in 0.1 M phosphate buffer (pH 7.4) and were dehydrated in graded acetone solution. Critical point drying and gold sputter coating was carried out under

reduced pressure in an inert argon gas atmosphere (*Agar Sputter Coater P7340*, All India Institute of Medical Sciences, India). After sputter coating the tissues were examined under scanning electron microscope (*Leo 435VP*, *Zeiss*, OR, USA) operated at 15 - 25 kV.

For the estimation of H_2O_2 the protocol of Zhou *et al*. (2006) was adopted. The procedure for the estimation of oxidized glutathione (GSSG) was according to O'Kane *et al*. (1996).

The day when the plantlets were first transferred from culture tubes to plastic cups in polyhouse was considered as control (zero month) and observations were taken after 1, 4 and 6 months. The experiments were repeated thrice independently, with at least twelve replicates for each treatment, except for the H_2O_2 and GSSG estimation, where the number of replicates was three. For statistical evaluation, *ANOVA* and least significant difference (LSD) (*Sigma Stat*, *SPSS*, Chicago, USA) at $P < 0.05$ were used.

Results and discussion

After four weeks of culture, maximum number of shoots was observed when 10 μM cPTIO and 10 μM NAA was supplemented in the modified MS medium (Table 1). Number of leaves decreased with increasing concentrations of cPTIO when supplied separately, however, this effect of cPTIO was ameliorated when 10 μM NAA was added (Table 1). The number of roots did not show significant changes in comparison with control when increasing concentrations either of cPTIO or 10 μM NAA was supplied in the medium, but the rooting was totally inhibited when NAA and cPTIO were given together. Almost 80 % of the cultures showed deformation in shoot growth when cPTIO was added to the medium either separately or in combination with NAA (Table 1).

Absence of Mg^{2+} and NAA led to the growth of knotted shoots with poor leaf initiation (Fig. 1*A*). A few globular bud like structures were observed arising from the shoot bases (Fig. 1*A*). Longitudinal sections of such globular bud like structures, revealed it as a mass of highly vacuolated cells with patches of provascular strands (Fig. 1*B*). Probably the knotted appearance and the globular bud like structures were due to inhibition of elongation growth. The ultrastructure of cortical cells of such knotted shoot buds, showed a progressive disorganization and disintegration of the chloroplast membranes with numerous vesicles and lamellae (Fig. 2*A*). The chloroplast stroma cannot be distinguished from the surrounding cytoplasm and highly metabolically active vacuole was mostly observed in cells deprived of Mg^{2+} and NAA (Fig. 2*A*). Accumulation of osmiophilic globules and the progressive disruption of the chloroplast membranes clearly indicated the cellular response of Mg^{2+} deficiency. Mg^{2+} is a key element for cell metabolism and its deficiency decreased CO_2 fixation and chlorophyll content and increased reactive oxygen species (ROS) production and accumulation of protoporphyrin XI (Cakmak and Marchner 1992, Esfandiari *et al*. 2010).

Table 1. Effect of NAA and cPTIO on shoot buds when supplemented to MS modified medium either separately or in combination after four weeks of culture. Means ± SE, $n = 12$. * - data significantly different from their respective control at $P < 0.05$.

NAA [μM]	cPTIO [μM]	Number of shoots	Number of leaves	Number of roots	Deformed shoots [%]
0	0	4.3 ± 0.2	4.7 ± 0.2	1.0 ± 0.1	30
10	0	4.9 ± 0.4	5.3 ± 0.3	1.5 ± 0.1	23
0	10	4.6 ± 0.2	3.4 ± 0.2*	0.3 ± 0.1	80
0	20	4.4 ± 0.2	2.6 ± 0.2*	0.2 ± 0.1	80
0	30	3.0 ± 0.4*	2.0 ± 0.3*	0.2 ± 0.1	80
10	10	5.8 ± 0.3*	4.5 ± 0.3	0	80
10	20	5.1 ± 0.4*	4.2 ± 0.2	0	80
10	30	4.0 ± 0.4	4.0 ± 0.3*	0	80

With the addition of 10 μM NAA in the medium, the shoots exhibited differentiation of leaves along with branched velamenous roots, termed here as coralloid roots which were agravitropic and did not penetrate into the medium (Fig. 1*C*). Longitudinal section of such roots revealed air spaces and starch granules (Fig. 1*D*).

Since auxin is required for several growth related responses, we hypothesize that fortification with exogenous NAA promoted leaf differentiation in our study. The absence of Mg^{2+} could possibly be the reason for both knotted shoots and agravitropic coralloid roots. Thimann and Biradivolu (1994) reported that Mg^{2+} is more effective than Ca^{2+} in promoting polymerization of actin, which occurs only in the presence of auxins.

Unlike IAA, NAA has greater passive permeability in the NAAH form (Rubery 1977), which appears to be a good form for polar transport as a whole (Hertel *et al*. 1969) and for the connection with auxin efflux carrier (Depta and Rubery, 1984). It is well reported by Allan and Rubery (1991), that in presence of NPA, a polar auxin transport inhibitor, Mg^{2+} inhibits less auxin efflux

than Ca^{2+}.

Further, we conducted two experiments applying TIBA, another polar auxin transport inhibitor. In the first set up, 10 µM NAA plus 10 µM TIBA in presence of Mg^{2+} and in absence of Ca^{2+} in MS medium was taken. The second set up comprised of 10 µM NAA plus 10 µM TIBA in presence of Ca^{2+} with the omission of Mg^{2+} from the medium. After 4 weeks of culture, the shoots without

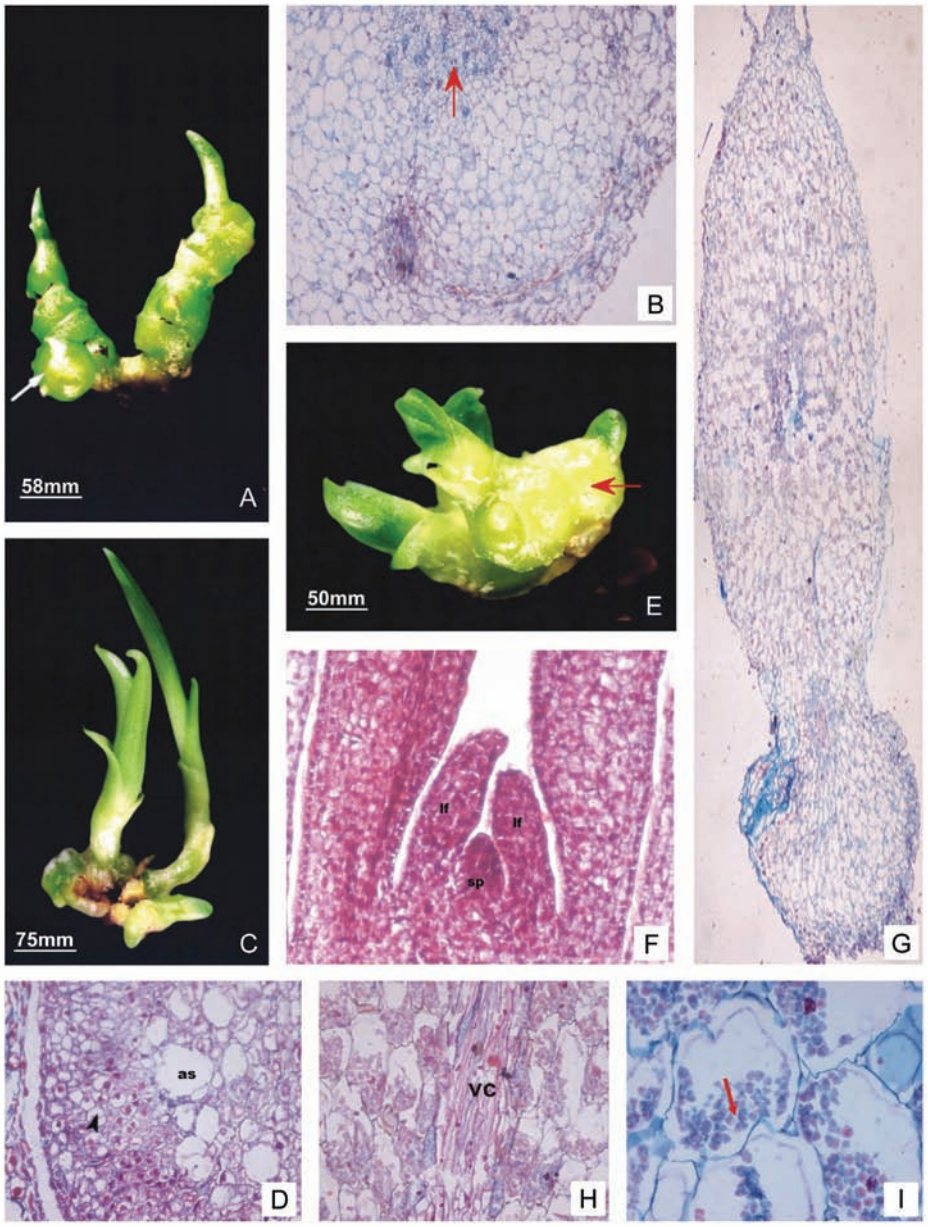

Fig. 1. Effect of NAA and a specific NO scavenger, cPTIO, in modified MS medium without Mg^{2+} on shoot buds after four weeks of culture. *A* - Knotted shoots with incipient leaf initiation and globular tumor like growth (*arrow*) arising from the shoot base of control shoots. *B* - Longitudinal section of the tumor like structures showing vacuolated cells and the *arrow* indicates a cluster of provascular strands. *C* - Shoots with leaves showing proper initiation and a clump of roots termed coralloid in 10 µM NAA supplemented medium. *D* - Longitudinal section of a portion of coralloid root showing large air spaces (as) and starch granules (*arrow head*) in 10 µM NAA supplemented medium. *E* - Leaves initiating from stout shoot buds emanating from undifferentiated tumor like structure at the shoot base (*arrow*) when treated with 10 µM NAA and 10 µM cPTIO. *F* - Longitudinal section of the stout shoot buds growing from tumerous shoot base showing properly developed shoot primordia (sp) with deeply stained apical meristem flanked by leaf initials (lf) when treated with 10 µM NAA and 10 µM cPTIO. *G* - Longitudinal section of knob like shoot (reconstructed) showing vacuolated cells with densely stained deposits when treated with 10 µM cPTIO. *H*, *I* - Longitudinal section showing densely stained deposits (*arrow*) along with vascular cylinders (vc) when treated with 10 µM cPTIO.

Ca^{2+} and in presence of Mg^{2+} + NAA + TIBA, did not show any trace of root formation (data not shown) and shoots were knotted. Conversely, in absence of Mg^{2+} and presence of Ca^{2+} + NAA + TIBA, a few coralloid roots with proper leaf initials were observed. Thus, we can conclude that Ca^{2+} is required to initiate the process of shoot formation in presence of polar auxin transport inhibitor (TIBA) and in presence of auxin (NAA). Therefore, our experiment clearly indicates the important role of NAA in agreement with Allan and Rubery (1991).

The specific NO scavenger cPTIO, added at three different concentrations (10, 20 and 30 μM) into the modified MS medium, induced stunted shoots without proper leaf initiation after 4 weeks of culture, which appeared as knob-like or pin-head like structures. Longitudinal sections of such knob like shoots observed in 10 μM cPTIO, did not show shoot apical meristem (Fig. 1*G*). In addition, cells were less deeply stained and instead filled with densely stained depositions (Fig. 1*I*) and vascular cylinders (Fig. 1*H*). Orchid cells are rich in mucilages (Arditti 1992) and, according to our opinion, these densely stained depositions formed due to binding of excess cPTIO with the mucilages. This is in accordance with the observation that the cells not treated with cPTIO did not show any such depositions. Our study is in agreement with Wright and Northcote (1974), who observed that mucilages have high capacity to bind aluminum.

Surprisingly, when 10 μM NAA plus 10 μM cPTIO was supplemented into the medium, stout shoots with leaves at different stages of initiation and undifferentiated tumor like growth at the shoot bases were noted (Fig. 1*E*). A well defined shoot primordium flanked with leaf initials were evident from the longitudinal sections of such tumorous structures (Fig. 1*F*). In this regard, cPTIO did not inhibit NAA in promoting differentiation only when used in equimolar concentrations, but at increasing concentrations of cPTIO (20 and 30 μM) reduction in the number of shoots was observed. Besides, there was a dramatic increase in the percentage of deformed shoots which rose up to 80 % of cultures with increasing concentrations of cPTIO in presence of 10 μM NAA, in comparison to control (Table 1). Similar findings were reported by Pagnussat *et al*. (2002), who observed that cPTIO delayed adventitious root emergency and significantly reduced the root length and number of roots in IAA treated explants and this inhibitory action of cPTIO was considered to be reversible by application of 200 μM SNP after cPTIO treatment.

Shoot buds were treated with different concentrations of SNP (10, 15 and 20 μM) and after 1 week of culture they showed a dose dependent increase in percentage of shoots exhibiting necrosis (Table 2). Ultrastructure of the cortical cells of shoots treated with 15 μM SNP, displayed numerous oil droplets, endocytosis of intact mitochondria bound by cytoplasmic membrane and internalization of membrane bound cytoplasmic inclusions in the central vacuole (Fig. 2*B*).

Table 2. Effect of SNP on H_2O_2 and GSSG contents and necrosis in shoot buds after one week of culture on modified medium. Means ± SE, n = 12. * - data significantly different from their respective control at $P < 0.05$.

SNP [μM]	H_2O_2 [μmol g^{-1}(f.m.)]	GSSG [μmol g^{-1}(f.m.)]	Necrosis [%]
0	0.081 ± 0.003	0.080 ± 0.002	5
10	0.189 ± 0.001*	0.088 ± 0.005	35
15	0.310 ± 0.002*	0.090 ± 0.004	60
20	0.285 ± 0.004*	0.098 ± 0.004*	80

Selga *et al*. (2005) reported that the ultrastructural mechanisms of removal of damaged or superfluous mitochondria in the central vacuole is the usual process during programmed cell death. The possibility of the presence of functionally inactive mitochondria in our study may be the reason for their internalization into the vacuole. This conclusion is mainly based on the observation that the existing mitochondria were seen to undergo repeated budding (by fission). In addition, mitochondria in some of the cells were highly elongated prior to budding, besides, numerous round and small mitochondria were noted after one week exposure to 15 μM of SNP. In this aspect our observations are similar to that of Sheahen *et al*. (2005), who noted that before protoplasts first divided, mitochondria underwent a phase of extensive elongation before fission, which caused an increase in number, followed by actin filament dependent distribution of mitochondria uniformly in the cytoplasm. Probably NO acted as a key messenger to activate the mitochondrial biogenesis program in various cell types (Nisoli and Carruba 2006).

In addition, we observed numerous oil droplets even in absence of NAA, which may be due to *de novo* synthesized lipids. Kennedy *et al*. (1991) suggested that such lipid droplets acted as electron and proton acceptors. Similar observations were made by Fox *et al*. (1994), who supposed lipid droplets as one of mechanisms of biochemical adaptation to anoxia. Tissue anoxia or hypoxia of *in vitro* grown plants can be due to high moisture content in the culture vessels, but it was precluded in our experiment by bottom cooling.

The responses like shoot necrosis and senescence of leaves after immediate exposure of SNP within one week was NO mediated. The reason for such conclusion was the dose dependent shoot necrosis when treated with increasing concentrations of SNP (Table 2). The shoot necrosis was due to oxidative stress imposed by SNP by generating toxic contents of H_2O_2. There was an increase in the concentration of H_2O_2 and hardly any change in the contents of GSSG with respect to control after one week of culture (Table 2). The gradual loss of pigments (an inherent symptom of leaf senescence) and necrosis in the leaves may be the consequences of enhanced generation of ROS (H_2O_2). H_2O_2 interrupts Calvin cycle at the

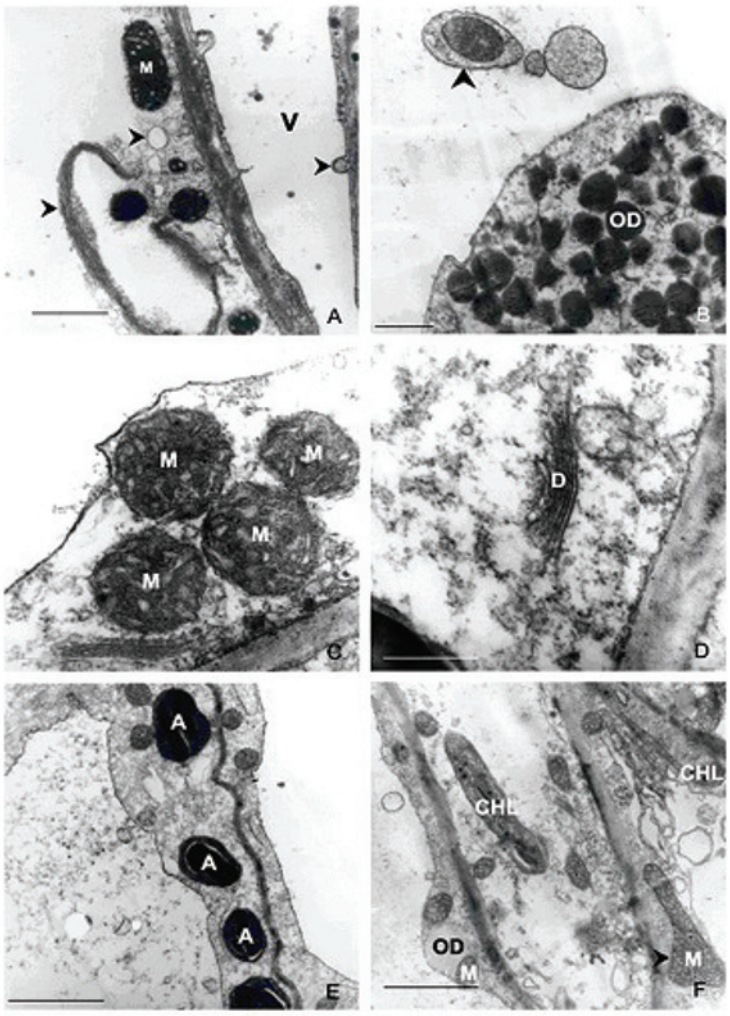

Fig. 2. Effect of SNP as NO donor on ultrustructure of cortical cells of shoot buds in MS medium without Mg^{2+}. *A* - Transmission electron micrograph showing active vacuole (V) with vesicles at the process of pinching into the vacuole (*arrow head*), mitochondria (M), lamellae (*arrow head*) and starch vesicles (*arrow head*) of disrupted chloroplast in the knotted shoots in control medium (without NAA, SNP, cPTIO and Mg^{2+}; *bar* = 1 μm). *B* - Membrane bound mitochondria (*arrow*) with some cytoplasmic inclusion and some portion of membrane bound cytoplast in the vacuole along with numerous oil droplets (OD) in the cytoplasm in cortical cells of necrosed shoot bud treated with 15 μM SNP after one week of culture (*bar* = 1 μm). *C*,*D* - Cluster of four mitochondria (M), which were round-shaped with prominent intercristal spaces, and a prominent dictyosome (D) can be seen in regenerating neomorphs treated with 15 μM SNP (*bar* = 1 μm). *E* - Four amyloplasts (A) and small round mitochondria along with two vacuoles in 15 μM SNP treated shoots (*bar* = 1 μm). *F* - Mitochondria (M) of varied shapes and sizes ranging from round to T-shaped (*arrow*) along with elongated chloroplasts (CHL) with plastoglobuli (black spots) and starch grains can be seen with few oil droplets (OD) in 15 μM SNP treated regenerating neomorphs (*bar* = 1 μm).

transketolase step, leading to reduced supply of the CO_2 acceptor ribulose 1,5-diphosphate (Kaiser 1976). In addition, H_2O_2 and NO have been identified as key molecules regulating ABA-induced stomatal closure in several plant species (Desikan *et al*. 2004). In *Tagetes erecta*, NO and H_2O_2 played a crucial role in the adventitious root development both synergistically and independently (Liao *et al*. 2009). NO is intimately linked with ROS as reported by Wendeheme *et al*. (2004), and NO reacts with $O_2^{\cdot-}$ producing peroxynitrite ($ONOO^-$), which itself is reactive and toxic. Therefore, NO acted as a pro-oxidant in our system after one week of culture.

De-differentiated shoot buds observed when treated with SNP after one week of culture, were re-differentiated into cream nodular structures termed ‘neomorphs’ after four weeks of culture. Neomorphs were anatomically and morphologically distinct from the protocorm like bodies (PLBs). They neither showed any marked shoot apical meristem nor were covered with tufts of rhizoids, a prominent feature of PLBs. The number of neomorphs increased with increasing concentrations of SNP (Table 3). Similarly, the number of rhizomes increased with the increasing concentrations of SNP and maximum number of rhizomes was observed in 15 and 20 μM of SNP (Table 3). Further, with increase in the concentration of cPTIO there was a rise in H_2O_2

Table 3. Effect of SNP and cPTIO on H_2O_2 and GSSG contents, number of neomorphs and rhizomes after four weeks in the second subculture. Means ± SE, $n = 12$. * - data significantly different from their respective control at $P < 0.05$.

SNP [μM]	cPTIO [μM]	H_2O_2 [μmol g^{-1}(f.m.)]	GSSG [μmol g^{-1}(f.m.)]	Number of neomorphs	Number of rhizomes
0	0	0.12 ± 0.02	0.097 ± 0.001	0	0
10	0	0.27 ± 0.05	0.158 ± 0.002*	14.3 ± 0.9*	5.1 ± 0.4
15	0	0.38 ± 0.03	0.166 ± 0.001*	33.8 ± 1.7*	7.3 ± 0.8*
20	0	0.44 ± 0.05*	0.183 ± 0.002*	25.8 ± 1.8*	6.5 ± 0.5*
0	0	0.15 ± 0.01	0.094 ± 0.001	-	-
10	10	0.30 ± 0.05	0.119 ± 0.001	-	-
10	20	0.41 ± 0.05*	0.137 ± 0.001*	-	-
10	30	0.52 ± 0.05*	0.140 ± 0.001*	-	-

generation. SNP-treated shoots also showed an increase in H_2O_2 generation at 20 μM concentration, but simultaneously there was also an increase in the GSSG activity (Table 3). Therefore, the increase in the GSSG activity possibly masked the toxic effect of H_2O_2 in SNP treated shoots. There was a slight increase in the activity of GSSG also with increasing concentrations of cPTIO, but GSSG activity became stable at 20 and 30 μM cPTIO treated shoots and less in comparison to SNP treated shoots (Table 3). Hence, it is possible that the oxidative stress was ameliorated in SNP treated shoots but not in the cPTIO treated shoots.

NO protects plant cells against oxidative stress by stimulating synthesis glutathione reduced form (GSH; Innocenti *et al*. 2007). GSH can readily react with NO to form GSNO, which serves as a NO reservoir in mammals (Zhang and Hogg 2004). Therefore, we suggest that NO mediated significant increase in GSSG activity masked the toxicity of H_2O_2 and promoted redifferentiation of neomorphs in our system.

The shoots when treated with 15 μM SNP turned into a globular cream structure and dead leaves were found still attached at its base (Fig. 3*A*). In addition, numerous cream neomorphs were also observed emanating from the shoot base (Fig. 3*A*). In most of the cultures treated with 15 μM SNP, development of rhizomes penetrating into the medium was observed (Fig. 3*B*). Surprisingly, a neomorph located at the tip of the rhizome was observed (Fig. 3*B*), which is usually the position for a terminal shoot in nature. Scanning electron micrographs of such neomorph on rhizome tip clearly indicated the presence of scale leaves with stomata (Fig. 3*D*) and some hairs on the surface (Fig. 3*C*). The formation of neomorphs after four weeks of culture clearly indicated a growth reversion. We suggest a role of NO in the initiation of re-differentiation process in our study. Transmission electron micrographs of the cortical cells of the neomorphs on the rhizome tips revealed the presence of amyloplasts (Fig. 2*E*) and several round mitochondria which were highly vacuolated (Fig. 2*E*). It is reasonable for the rhizome to produce plenty of starch, which would provide energy and be a mean for gravitropism (Audus 1962). Recently, Wang *et al*. (2010a) reported high expression of metabolism-related genes in rhizomes. The expression of starch accumulation related genes was notably high in the rhizome buds of bamboos (Wang *et al*. 2010a).

After four weeks of culture, neomorphs treated with three different concentrations of SNP showed re-greening and regeneration. Maximum greening of neomorphs followed by maximum percentage of neomorphs regenerating into shoots were observed in 15 μM SNP supplemented medium (Table 4). Furthermore, maximum number of axillary shoot buds emanating from the axil positions of rhizomes were observed, when treated with 15 μM SNP (Table 4). However, there was a sharp decline in the number of axillary shoot buds and in the number of green neomorphs when the concentration of SNP was raised up to 20 μM (Table 4). In contrast, the neomorphs treated by cPTIO at three different concentrations showed negligible greening (Table 4).

After four weeks of culture, the SNP treated neomorphs turned into deep green regenerating shoot buds and most of them exhibited properly developed leaf initials (Fig. 3*E*). Greening of neomorphs is mainly due to NO because cPTIO treated neomorphs did not show greening. Recently, Kumar *et al*. (2010) reported that SNP was more effective than other NO donors in causing re-greening of leaves of iron-deficient maize plants and increased *in vivo* NO content, also raised chlorophyll and carotenoid contents.

The rhizome apex, noted as a neomorph, fully developed into a shoot. Several axillary shoot buds were seen emanating from the axils of one such rhizome (Fig. 3*F*). On an average 10 shoot buds were formed on one rhizome when treated with 15 μM of SNP. Transmission electron micrographs showed clusters of round shaped mitochondria with prominent intercristal spaces (Fig. 2*C*) in shoots treated with 15 μM SNP. Fully active dictyosomes were also seen with several vesicles in its vicinity (Fig. 2*D*), clearly indicating a role of NO in the process of regeneration. In contrast, Lehner *et al*. (2008) observed NO induced functionally inactive dictyosomes in unicellular green alga *Micrasterias denticulata*. Different shapes of mitochondria, several elongated chloroplasts with plastoglobuli (appearing as black spots) and starch grains were observed in regenerating neomorphs in 15 μM SNP treated shoot

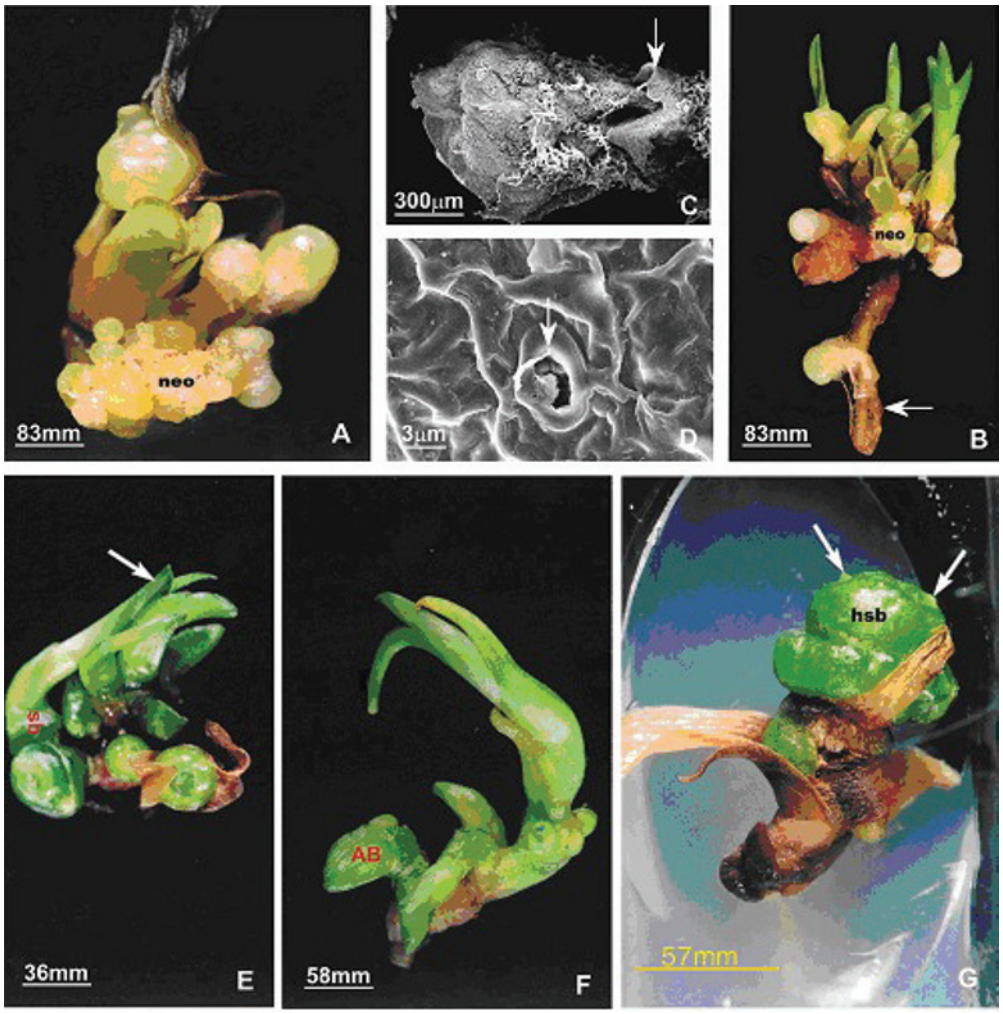

Fig. 3. Effect of SNP and cPTIO supplemented in MS medium without Mg^{2+} on neomorph formation and regeneration of plantlets after four weeks of culture. *A* - Shoots turned into a cream swollen globular structure with necrotic leaves and numerous cream neomorphs (neo) arising at the shoot base when treated with 15 µM SNP. *B* - Shoots were converted to globular neomorphs (neo) after shedding of leaves. Note the coiled rhizome (*arrow*) with a neomorph at its tip when treated with 15 µM SNP. *C*,*D* - Scanning electron micrographs of the rhizome showing presence of scale leaves (*arrow*) and also the presence of a stomata on the neomorph (*arrow*) when treated with 15 µM SNP. *E* - Deep green neomorphs converted to shoot bud with proper initiation of leaves (*arrow*) was witnessed on the neomorph situated at the tip of rhizomes in SNP treated shoots. *F* - Well developed rhizome with leafy shoot at its tip and axillary bud (AB) obtained by SNP treatment. *G* - Hypertrophied shoot bud (hsb) with incipient leaf initials (*arrows*) were seen when sub-cultured from 10 µM cPTIO on pre-hardening medium.

Table 4. Effect of SNP and cPTIO supplemented to MS medium without Mg^{2+} on number of yellow and green neomorphs, their conversion to shoots and number of axillary buds after four weeks of culture on shoot buds. Means ± SE, n = 12. * - data significantly different from their respective control at $P < 0.05$.

SNP [µM]	cPTIO [µM]	Number of yellow neomorphs	Number of green neomorphs	Conversion to shoots [%]	Number of axillary buds
0	0	12.4 ± 1.1	1.3 ± 0.1	10	2.7 ± 0.3
0	10	13.3 ± 1.4	1.7 ± 0.2	0	1.3 ± 0.1
0	20	14.6 ± 1.8	0	0	0
0	30	18.3 ± 2.0	0	0	0
10	0	9.6 ± 1.1	40.8 ± 3.2*	85	7.2 ± 1.9*
15	0	7.0 ± 0.5*	43.2 ± 2.8*	85	10.2 ± 2.0*
20	0	15.7 ± 2.8	36.3 ± 1.2*	85	6.0 ± 0.5*

Table 5. Acclimatization of SNP induced plantlets under *ex vitro* conditions in polyhouse at different periods. Means ± SE, n = 12, * - significant differences from the beginning of acclimatization.

Time [months]	Number of roots	Root length [cm]	Leaf length [cm]	Leaf width [cm]	Number of axillary shoots
0	2.0 ± 0.4	1.1 ± 0.5	1.6 ± 0.2	0.2 ± 0.1	1.1 ± 0.2
1	7.8 ± 1.5*	1.7 ± 0.2	2.7 ± 0.3*	0.6 ± 0.4	1.1 ± 0.3
4	12.0 ± 1.5*	2.4 ± 0.2	3.3 ± 0.3*	0.9 ± 0.4*	3.0 ± 0.2*
6	13.3 ± 1.1*	3.6 ± 0.4*	3.4 ± 0.2*	1.2 ± 0.1*	3.5 ± 0.2*

buds (Fig. 2*F*). The structure-function relationship between mitochondrial morphology and energy-producing activity was mentioned, *e.g.*, by Nisoli and Carruba (2006).

Therefore, we firmly suggest that the process of de-differentiation (indicated by shoot necrosis into formation of neomorphs) and re-differentiation (represented by regeneration of neomorphs to green shoots and rhizomes) is due to SNP as NO donor under Mg^{2+} deficient conditions in our system. We believe that our results have provided enough evidence to support the role of NO in this regard.

The transplantation stage continues to be a major bottleneck in propagating plants under *in vitro* conditions. Most species grown *in vitro* require an acclimation process in order to ensure that sufficient number of plants survives in field conditions. Therefore the plantlets obtained by re-differentiation of neomorphs of SNP treatments were further sub cultured to the pre-hardening medium.

After four weeks of culture, regenerated shoot buds previously treated by SNP developed into plantlets with one or two velamenous roots. However, 10 μM cPTIO induced neomorphs, when sub-cultured in pre-hardening medium, developed into hypertrophied shoot buds with two incipient leaf initials (Fig. 3*G*).

The plantlets obtained in the pre-hardening medium were transferred to polyhouse for acclimatization. There was an increase in the number of roots in the first, fourth and sixth months. Maximum increase in root length was observed on 6-month-old plants (Table 5). Leaf length started to increase from 1-month-old plants. Both, leaf width and number of axillary shoots emanating from rhizomes increased in 4- and 6-month-old plants (Table 5).

In conclusion, we highlight the principal results of this study with *Cymbidium*: *1*) NAA under Mg^{2+} deficient conditions cannot elicit rhizome and NO has no role in it; *2*) Exposure to SNP induced oxidative stress and it is responsible for leaf senescence and shoot necrosis; *3*) De-differentiation of mature shoot tip to a neomorph and re-differentiation of the neomorph to plantlets and rhizome is due to NO and its interaction with antioxidant like GSH.

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