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EFFECT OF ORGANIC AND INORGANIC NITROGEN SOURCE ON SHOOT REGENERATION AND HYPERHYDRICITY IN *TECOMELLA UNDULATA* (SM.) SEEM DURING MICROPROPAGATION

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ABSTRACT: *Tecomella undulata* is one of the most important agroforestry species of arid region. The effects of different concentrations of NH_4NO_3, KNO_3, NH_4NO_3 X KNO_3 and Glutamine were investigated on proliferation stage of *T. undulata* cultivated on MS media as nitrogen sources. The highest shoot length and multiplication was obtained with combination of NH_4NO_3 X KNO_3 (10.31 mM X 9.40 mM) with low hyperhydricity (10.3%). Overall, the MS N combination was superior to any single N source for proliferation and growth of shoots. The explants cultured with other nitrogen sources resulted in low culture frequency and low number of shoots per explant accompanied by basal callusing and hyperhydricity. This study also demonstrates that organic source of N as glutamine does not show any improvement in shoot growth and hyperhydricity.

KEYWORDS: Ammonium nitrate, Glutamine, Micropropagation, Multiplication rate, Hyperhydricity, MS medium

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1. INTRODUCTION

The performance and survival of *in vitro* cultures of many plant species are often hampered by the phenomenon of hyperhydricity (Deberg *et. al,* 1992). Hyperhydricity can lead to irreversible loss of regenerative ability of the tissue (Gaspar *et. al,* 2000) and other detrimental changes, and ultimately death. These losses, together with the poor survival rate of hyperhydric shoots (HS) when transferred

to *ex vitro* conditions, limit the potential of *in vitro* techniques for mass propagation (Ivanova and Van Staden 2008).It appears that hyperhydricity is induced by the combined action of several physical and chemical factors of the culture environment (Gaspar 1991; Ziv 1991; Debergh *et. al,* 1992). However, many of these factors would only act to induce hyperhydricity when other conditions in the culture system are not optimized (Deberg *et. al,* 1992). A number of investigations have been reported on the hyperhydricity-inducing tendency of exogenous cytokinins (CKs), usually in a concentration-dependent manner (Leshem *et. al,* 1988; Kataeva *et. al,* 1991; Williams and Taji 1991; Ivanova *et. al,* 2006). Large quantities of ammonium ions have also been shown to increase hyperhydricity in different species (Vieitez *et. al,* 1985; Daguin and Letouze 1986; Brand 1993). We have shown that the incidence of hyperhydricity in micropropagated shoots of *Tecomella undulata*, the species in the present investigation, is affected by the synergistic action of Ammonium Nitrate (NH_4NO_3) and Potassium Nitrate (KNO_3) levels. *Tecomella undulata* (Rohida) is a highly valuable and threatened species, restricted to the drier parts of the Arabia, southern Pakistan and northwestern India up to an elevation of 1200 meters (Tewari, 2007). The tissue culture media used for regeneration of *T. undulata* shoots (Rathore *et. al,* 1991; Bhansali *et. al,* 1993; Robinson *et. al,* 2005; Varshney *et. al,* 2011; Danaya *et. al,* 2012; Tyagi *et. al,* 2013; Kumari and Singh 2014) are based on the mineral nutrient composition formulated by Murashige and Skoog (1962), where typically the N pool of 60 mM is comprised of inorganic NH_4NO_3 and KNO_3. However, previously no study was conducted to provide a physiological basis for using this N concentration or ratio for the micropropagation of this species.The influence of inorganic NH_4NO_3 and KNO_3 and organic glutamine as a sole N sources as well as the concentration of NH_4NO_3 X KNO_3 were evaluated with the special emphasis placed on shoot regeneration, incidence of hyperhydricity and production of good quality shoots in tissue cultured *T. undulata.*

2. MATERIALS AND METHODS

Micropropagated shoots of *T. undulata* were established using single mature nodal explant (Rathore *et. al,* 1991). The culture was subsequently maintained and multiplied by subculturing the shoots at an interval of 4 weeks. They were grown on basal Murashige and Skoog (1962) medium (MS) supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol (Sigma, St. Louis, MO, USA), 0.057 µM IAA (Sigma) and, 4.43 µM BAP (Sigma). The pH was adjusted at 5.8 with the help of 1 N HCL and 1N NaOH. 0.8% agar (w/v) was used as a gelling agent. Autoclaving of the medium was done at 15 psi for 15-20 minutes at 121˚C temperature. The cultures were maintained under a continuous photoperiod in a growth room fitted with cool white-fluorescent tubes (Philips India) at 2500 lux intensity for 16h photoperiod, at 26 ± 2˚C, and a relative air humidity of approximately 60%. Shoots regenerated *in vitro* were used as initial explants in the present experiments.

Experimental design

In the first experiment, different sources of nitrogen compounds – NH_4NO_3 (0, 5.15, 10.31, 15.37, 20.61 mM) only, KNO_3 (0, 4.70, 9.40, 14.09, 18.79 mM) only and a combination of both were tested (Table 3). The basal MS medium was modified as follows: (i) KNO_3 salt was excluded from the medium andNH_4NO_3 act as sole source of nitrogen at different concentration in the medium; (ii) KNO_3 was act as sole source of nitrogen and NH_4NO_3 salt was excluded from the medium; (iii) Combination of both NH_4NO_3 and KNO_3 at different concentration; and (iv) In the final experiment both $NH4NO_3$ and KNO_3 were excluded from the medium and glutamine, as an organic source of N was tested at five concentrations: 0, 0.1, 0.5, 1.5 and 5.0 g l^{-1}. The MS basal medium did not contain any form of inorganic N.

Culture conditions

The basal media were prepared as described above for the various treatments, and supplemented with 30 g^{-1} sucrose, 100 mg l^{-1} myo-inositol (Sigma, St. Louis, MO, USA), 0.057 µM IAA (Sigma) and, 4.43 µM BAP (Sigma). The pH was adjusted at 5.8 with the help of 1 N HCL and 1N NaOH. 0.8% agar (w/v) was used as a gelling agent. Autoclaving of the medium was done at 15 psi for 15-20 minutes at 121˚C temperature. Explants, with an average length of 25 ± 5 mm (mean ± SD) and with five to six leaves were obtained from *in vitro* grown shoots. They were cultured in 250 ml tissue culture flasks, each containing 50 ml of medium. Three explants were planted per flask and each treatment comprised 15 flasks. Each experiment was repeated at least twice. The cultures were incubated at 26 ± 2˚C under a continuous photoperiod of 2500 lux intensity for 16h photoperiod.

Data collection and Statistical analysis

At the end of the 4-week culture period the number of shoots per explant (multiplication rate) was recorded. The newly formed shoots were categorized as normal shoots (NS) and hyperhydric shoots (HS), according to their external appearance and hyperhydricity (%) was calculated. HS shoots had thicker, translucent and water-logged leaves compared to NS. A complete randomized design was used in all experiments and analysis of variance (ANOVA) and means separation were carried out using Duncan's Multiple Range Test (DMRT) with significance determined at 5 % level using SPSS (version 17.0) software

3. RESULTS AND DISCUSSION

Effect of N source and concentration

Shoot proliferation was observed in all treatments, including when N was omitted from the culture media (0 mM), where an average of 1.1 multiplication rate was scored but shoots were pale green and very short (on average of 5.75 mm; Tables 1, 2, 3, 4).

Table 1. Effect of ammonium nitrate as the sole source of nitrogen in culture of *T. undulata* after 4 weeks

NH_4NO_3 conc (mM)	Hyperhydricity (%)[a]	Multiplication rate ± SE (shoots/explant)a	Mean increament in shoot length ± SE (mm)	Callus intensity
0	4.4[c]	0.7±0.1[d]	10.0±0.2[c]	-
5.15	8.7[bc]	1.6±0.1[c]	19.1±0.1[b]	+
10.31	11.2[bc]	2.6±0.1[a]	24.3±0.1[a]	+
15.37	24.5[b]	2.3±0.1[ab]	22.6±0.1[a]	++
20.61	46.3[a]	2.0±0.1[b]	21.2±0.2[a]	+++

[a]Means with the same letter within a column are not significantly different at $P<\_ 0.05$ when separated by DMRT

(-) = no callusing, (+) = very less callusing, (++) = moderate callusing, (+++) = high callusing

When NH_4NO_3 was used as a sole source of N, the multiplication rate was comparatively low, irrespective of the concentration applied (Table 1). However, the incidence of hyperhydricity and callusing was very high, reaching a maximum on media with 20.61 mM (46 % HS, respectively; Table 1). The quality of new shoots was poor with low multiplication rate and shoot length.High quality of new shoots with dark green leaves was produced on media supplemented with KNO_3 only as the N source. Replacing NH_4NO_3 with KNO_3 in the medium resulted in reduction of hyperhydricity and callusing and an increase in multiplication rate (Table 2). The highest multiplication rate (3.1) with maximum shoot length (30.6mm was obtained on KNO_3 (9.40 mM).

Table 2. Effect of potassium nitrate as the sole source of tissue cultured *T. undulata* after 4 weeks

KNO_3 conc (mM)	Hyperhydricity (%)[a]	Multiplication rate ± SE (shoots/explant)[a]	Mean increment in shoot length ± SE (mm)	Callus intensity
0	2.3 [e]	1.1 ± 0.09 [d]	11.2 ± 0.05 [c]	-
4.70	4.9 [d]	2.6 ± 0.13 [b]	14.8 ± 0.10 [c]	-
9.40	6.7 [c]	3.1 ± 0.19 [a]	30.6 ± 0.17 [a]	-
14.09	9.0 [b]	2.4 ± 0.11 [bc]	23.7 ± 0.14 [b]	+
18.79	12.1 [a]	2.1 ± 0.15 [c]	21.5 ± 0.14 [b]	+

[a] Means with the same letter within a column are not significantly different at $P<\_$ 0.05 when separated by DMRT

(-) = no callusing, (+) = very less callusing, (++) = moderate callusing, (+++) = high callusing

Effect of NH_4NO_3 and KNO_3 concentrations:

The highest multiplication rate was observed on the medium containing both NH_4NO_3 and KNO_3. One way ANOVA showed that different NH_4NO_3 with KNO_3 concentrations had significant effects on all parameters studied: multiplication, shoot length, hyperhydricity and callusing of *T. undulata* after 4 weeks in culture. The best shoot formation was obtained with NH_4NO_3 X KNO_3 (10.31 mM + 9.40 mM). The new shoots had healthy appearance with green leaves. A further increase in the concentration of NH_4NO_3 and KNO_3 led to significant decrease in shoot proliferation, shoot length and increase in hyperhydricity and callusing of the cultures. Hyperhydricity increased progressively with the increase of both nitrogen sources and reported maximum of 57% at concentration of NH_4NO_3 (20.61 mM) +X KNO_3 (18.79 mM) [Table 3]. The average length of long shoots decreased gradually when raising the concentration of NH_4NO_3 and KNO_3 [except for NH_4NO_3 (20.61 mM) X KNO_3 (18.79 mM); Fig. 1].

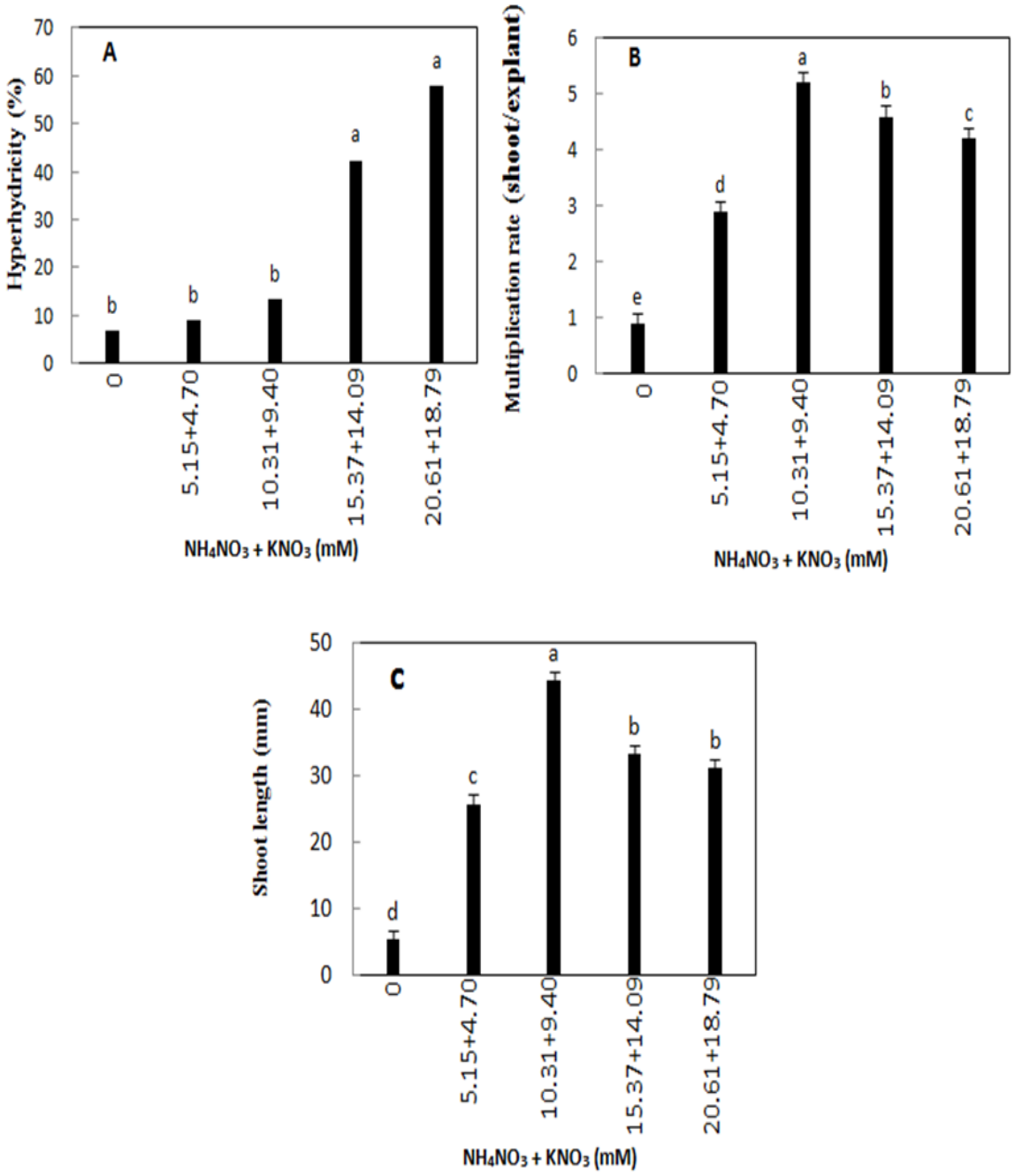

Fig 1, Effect of NH_4NO_3 and KNO_3 (mM) concentration in the culture media on the (A), hyperhydricity (B) shoot multiplication rate and (C) the mean shoot length of *T. undulata* after 4 week in culture. Bars with common letters are not significantly different at $p \leq 0.05$ according to DMRT

Table 3 Effect of ammonium nitrate and potassium nitrate on *in vitro T. undulata* after 4 weeks in culture

NH_4NO_3 + KNO_3 CONC (mM)	Hyperhydricity (%)[a]	Multiplication rate ± SE (shoots/explant)[a]	Mean increament in shoot length ± SE (mm)	Callus intensity
0	6.70 [b]	0.9 ± 0.06 [e]	5.2 ± 0.06 [d]	-
5.15 + 4.70	8.91 [b]	2.9 ± 0.10 [d]	25.6 ± 0.19 [c]	+
10.31 + 9.40	10.3 [b]	5.2 ± 0.06 [a]	44.3 ± 0.30 [a]	+
15.37 + 14.09	42.2 [a]	4.6 ± 0.11 [b]	33.2± 0.26 [b]	++
20.61 + 18.79	57.8 [a]	4.2 ± 0.15 [c]	31.1 ± 0.30 [b]	+++

[a] Means with the same letter within a column are not significantly different at $P<\_$ 0.05 when separated by DMRT (-) = no callusing, (+) = very less callusing, (++) = moderate callusing, (+++) = high callusing

Effects of Glutamine concentration: Poor quality of new shoots, with pale green leaves, were obtained on media with glutamine. Increasing the concentration of glutamine resulted in significant increase in hyperhydricity and callusing. In contrast shoot multiplication and shoot length was not affected significantly and retained relatively low rates, with marginal differences among the treatments (Table 4). The highest percentage of multiplication was obtained on media supplemented with 0.5 mg l^{-1} (3.0) or 1.5 mg l^{-1} (2.9) glutamine with maximum length of 28.9 (Table, 4).

Fig 2: Effect of MS media (A) and modified MS media (B) on shoot cultures of *T. undulata* after four weeks of subculturing

Table 4 Effect of glutamine as a sole source of nitrogen in culture of *T. undulata* after 4 weeks

Glutamine conc ($g\ l^{-1}$)	Hyperhydricity (%)[a]	Multiplication rate ± SE (shoots/explant)[a]	Mean increment in shoot length ± SE (mm)	Callus intensity
0	8.9 [d]	1.0 ± 0.10 [d]	13.2 ± 0.07 [c]	-
0.1	20.0 [cd]	1.4 ± 0.12 [c]	16.8 ± 0.10 [c]	+
0.5	35.5 [bc]	3.0 ± 0.11 [a]	28.9 ± 0.17 [a]	++
1.5	46.7 [ab]	2.9 ± 0.14 [ab]	21.5 ± 0.11 [b]	++
5.0	61.2 [a]	2.5 ± 0.20 [b]	20.7 ± 0.13 [b]	+++

[a]Means with the same letter within a column are not significantly different at $P<\_ 0.05$ when separated by DMRT

(-) = no callusing, (+) = very less callusing, (++) = moderate callusing, (+++) = high callusing

DISCUSSION

Nitrate has been regarded as the principal form of N for plant tissue cultures (Sathyanarayana and Blake 1994). In the present study NO_3^- as the sole source of N was sufficient for the proliferation and growth of new shoots. Similarly, when NO_3^- used as the single N source, successful regeneration was reported in various tissue culture systems (Cousson and Tran Thanh Van 1993; Tsai and Saunders 1999: Ramage and Williams 2002: Woodward *et. al,* 2006). Information on the effect of NO_3^- on hyperhydricity is scare. Nagabuko *et. al,* (1993) observed that no hyperhydricity occurred on a NO_3^- only medium (60 mM) used for garlic shoot proliferation. This finding corroborate our data that hyperhydricity and callusing was almost eliminated on media with NO_3^- as a sole source of N. Ammonium used as the sole source of N appeared to have a negative effect on regeneration and growth of new shoots of *T. undulata*. Although N assimilation is associated with reduction of NO_3^- to NH_4^+, many plant species showed inhibition of morphogenesis and growth when NH_4^+ was supplied as the exclusive N source: *Nicotiana tabacum* (Cousson and Tran Thanh Van 1993; Walch-Liu *et. al,* 2000; Ramage and Williams 2002), *Oryza sativa* (Grimes and Hodges 1990), *Solanum tuberosum* (Avila *et. al,* 1998) and *Eucalyptus marginata* (Woodward *et. al,* 2006). NH_4^+ induced growth depression of tobacco leaves was a result of both reduced cell division and cell elongation (Walch-Liu *et. al,* 2000). The inhibition of morphogenesis and growth in response to application of NH_4^+ as the sole N source has been attributed mainly to changes in medium pH and toxic effects of free NH_4^+. Ammonium nutrition is associated with acidification of the medium. Low pH affects the availability

of mineral nutrients in the medium, with most of them becoming limited when pH falls below 5 (Williams 1993) and therefore restricting explant growth. Ammonium supplied as NH_4NO_3 at 20.6 mM induced hyperhydricity in several studies (Vieitez *et. al,* 1985; Daguin and Letouze´ 1986; Brand 1993). The present study revealed that the MS N mixture was superior to any single N source for regeneration and growth of shoots. Other studies also found the MS N regime more efficient than a single N source (Avila *et. al,* 1998; Tsai and Saunders 1999; Ramage and Williams 2002). At a concentration of 60 mM (standard MS medium), N induced hyperhydricity in *Prunus avium* (Riffaud and Cornu 1981), *Castanea sativa* (Vieitez *et. al,* 1985), *Salix babylonica* (Daguin and Letouze´ 1986) and *Aloe. polyphylla* (Ivanova and van Staden 2008). However, the induction of hyperhydricity was attributed, by these authors, not to the N, but to the NH_4^+, which could trigger a series of events leading to this state (George 1993). Lowering the N to a half of its amount (30 mM) eliminated hyperhydricity (Riffaud and Cornu 1981; Chauvin and Salesses 1988). Our data corroborate these findings (Table 3). Analysis of variance showed that, shoot length significantly influenced by different concentrations of NH_4NO_3 X KNO_3 interaction (NH_4^+:NO_3^- ratio). The highest shoot length was observed in 10:20 ratio of NH_4^+:NO_3^-(10.31 mM + 9.40 mM). In this ratio the amount of total nitrogen in culture medium was reduced (30 mM). The lowest shoot length was observed in 5:10 NH_4^+:NO_3^- ratio (total N 15 mM). In comparison of 10:20 ratio; more concentration of ammonium ion and high total nitrogen may be the main reason of decreasing shoot length. The high level of ammonium ion may be inhibited growth of shoot length due to decrease in the activity of Nitrate Reductase and Glutamate Synthases Enzymes in producing of amino acids (Gamborg and Shyluk, 1970). So nitrate is the most important form of nitrogen that used by the *T. undulata.* Glutamine could not be used successfully as the exclusive N source for shoot production and growth of *T. undulata* it induces callusing as well as hyperhydricity in cultures. Glutamine was found to support callus initiation and subsequent shoot regeneration from sugar beet leaf discs (Tsai and Saunders 1999) as well as shoot tip culture of cucumber (Vasudevan *et. al,* 2004). In conclusion, N is an essential nutrient for regeneration and development of *T. undulata* shoots, such that almost no proliferation and growth occurred in the absence of N. The results presented here clearly indicate the diverse response to the various forms of N supplied, reflecting the previously overlooked importance of this nutrient in determining plant morphology and morphogenesis *in vitro*.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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