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Original Article

Range of factors in the reduction of hyperhydricity associated with *in vitro* shoots of *Salvia santolinifolia* Bioss

Gama de fatores na redução da hiperidricidade associada a brotos *in vitro* de *Salvia santolinifolia* Bioss

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Abstract

Hyperhydricity is a serious physiological disorder and affects *In vitro* propagation of many plants and as well of *Salvia santolinifolia*. The donor material to initiate the *in vitro* culture was the callus taken from the *in vitro* shoots produced on Murashig and Skoogs (MS) medium at 4.0 mg/l BA. This callus formed numerous hyperhydric shoots on culturing upon the medium of the same composition. The aim was to systematically evaluate the effect of cytokinins (Benzyladnine (BA) and N^6-(-2-isopentenyl) adenine (2iP), culture vessels magnitude, medium solidification, source of nitrogen and calcium chloride for the alleviation of hyperhydricity. In the tissue cultures of *S. santolinifolia* BA and 2iP induced severe hyperhydricity, when other factors i.e. culture vessels magnitude and a suitable concentration of agar, ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3) & calcium chloride ($CaCl_2.2H_2O$) were not optimized. After 30 days' culture, we observed 83.82% hyperhydric shoots at increased level (1.5 mg/l 2iP) and 81.59% at decreased levels (1.0 mg/l 2iP). On the other hand, hyperhydricity percentage at decreased (0.4%) and at increased (0.8%) levels of agar were 72.37% and 39.08%, respectively. MS medium modification with NH_4NO_3 (412 mg/l), KNO_3 (475 mg/l) and $CaCl_2.2H_2O$ (880 mg/l) was found the best medium to reduced hyperhydricity (23.6%).

Keywords: agar, culture vessels, growth regulators, macronutrients, lamiaceae.

Resumo

A hiperidricidade é um distúrbio fisiológico sério e afeta a propagação *in vitro* de muitas plantas e também da *Salvia santolinifolia*. O material doador para iniciar a cultura *in vitro* foi o calo retirado dos brotos *in vitro* produzidos em meio Murashig e Skoogs (MS) a 4,0 mg / l BA. Esse calo formou numerosos rebentos hiperídricos em cultura no meio da mesma composição. O objetivo foi avaliar sistematicamente o efeito das citocininas (Benziladnina (BA) e N6 - (- 2-isopentenil) adenina (2iP), magnitude dos vasos de cultura, solidificação do meio, fonte de nitrogênio e cloreto de cálcio para o alívio da hiperidricidade. culturas de tecidos de *S. santolinifolia* BA e 2iP induziram hiperidricidade severa, quando outros fatores, como magnitude dos vasos de cultura e uma concentração adequada de ágar, nitrato de amônio (NH4NO_3), nitrato de potássio (KNO_3) e cloreto de cálcio (CaCl2.2H_2O), não foram otimizados. Após 30 dias de cultura, observamos 83,82% de brotos hiperídricos em níveis aumentados (1,5 mg / l 2iP) e 81,59% em níveis reduzidos (1,0 mg / l 2iP). Por outro lado, a porcentagem de hiperidricidade diminuiu (0,4%) e em níveis aumentados (0,8%) de ágar foram 72,37% e 39,08%, respectivamente. A modificação do meio MS com NH_4NO_3 (412 mg / l), KNO_3 (475 mg / l) e CaCl2.2H2O (880 mg / l) foi encontrada melhor hiperidricidade média a reduzida (23,6%).

Palavras-chave: ágar, vasos de cultura, reguladores de crescimento, macronutrientes, lamiaceae.

1. Introduction

Hyperhydricity is a physiological disorder and hampered the micropropagation of many plants. Phillips and Matthews (1964) reported it for the first time in carnation shoot tip culture. Hyperhydric individual obstructs the industrial production of plants regeneration and caused economic loss. For nearly 60% of plants species, hyperhydricity was described to results in a great loss (Tabart et al., 2015; Tian et al., 2017). Hyperhydricity are

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often affect the performance and persistence of tissue culture of many plant species (Debergh et al., 1992) and can lead in to permanent damage of regenerated capability of the tissue (Gaspar et al., 2000) and the conservation of endangered species became more problematic (Pence et al., 2014), hence the inhibition of this difficult is very essential. Numerous factors can encourage the hyperhydricity an *in vitro* shoots, large quantities of ammonium ions (Brand 1993), high level of exogenous cytokinins (Ivanova et al., 2006; Oliveira et al., 2010), vessel aeration (Lai et al., 2005), cultivar (Carvalho et al., 2013) and agar concentrations in the medium (Abdoli et al. 2007). The greatest collective reason of the hyperhydricity existence is the culture in liquid medium (Silva et al., 2013) and the use of high concentrations of cytokinins causes the accumulation of ethylene in the culture vessels (Isah, 2015).

Salvia is one of the well-known genus of family Lamiaceae with round about 800 species. Genus *Salvia* is well known for its anti-tumor phytochemicals (Ginda and Kakisawa, 1990). In the frequent exploration for novel bioactive compounds one new and two know compounds were isolated from the callus culture of *S. santolinifolia* (Jan et al., 2018). The methods of tissue culture controls the conservation of plant genetic properties without depleting their natural place; because it requires a small fragment of plants to initiate the regeneration process (Mikula and Rybczynski, 2006). During the callus culture of *S. santolinifolia*, hyperhydricity easily burst on a large scale and causes high fatalities in term of effort, material and financial resources. To study the scope of factors on hyperhydricity of *S. santolinifolia*, we evaluated the effects of cytokinin types and concentration, culture vessels magnitude, medium solidification and source of nitrogen with calcium chloride on hyperhydricity and regeneration of adventitious shoots from callus. This study contributes to our understanding of the effect of factors on hyperhydricity in shoots from callus of *S. santolinifolia*. Developing an effective system of regeneration would be a useful tool for the conservation of this species and others members of the family Lamiaceae.

2. Materials and Methods

2.1. Experimental materials and subcultures

The donor explants (nodes and leaves) for the initiation of callus was obtained from 90 days old *In vitro* shoots on MS medium augmented with BA (4.0 mg/l) BA+NAA (4.0+0.1 mg/l), sucrose (3%), agar (0.5%), pH (5.55-5.56), (Table 1) in the initial experiment. The nodes derived callus showed organogenic potential and was selected for further experiments for the induction of adventitious shoots while callus of leaves failed to form adventitious shoots and was not used in further experiments.

The callus and hyperhydric shoots formed after 30 days of incubation were subcultured on MS media containing different concentrations of BA (3.0 and 3.5 mg/l) and 2iP (1.0 and 1.5 mg/l) for induction of multiple shoots (Table 2). The hyperhydric shoots along with callus were also shifted to MS medium supplemented with combination of cytokinin and gibberellin (BA+GA_3 (3.0+2.0 and 3.5+2.0 mg/l) and 2iP+GA_3 (1.0+2.0 and 1.5+2.0 mg/l) (Table 3).

Table 1. Induction of callus in the influence of Cytokinin and Auxin.

Hormones (mg/l)	Explants	% response	Nature of callus
BA 4.0	*In vitro* nodes	90-100	Whitish, granular and friable
BA/NAA 4.0/0.5	*In vitro* Leaf	55-82	Yellowish and friable

Table 2. The influence of BA and 2iP on hyperhydricity in subculture in small size container (90×51mm).

Hormones (mg/l)	%HS	LHS (cm)	Nature of hyperhydric shoots	
			Shoots	Leaf
BA				
3.0	86.72	0.79	Yellowish green, internodes short, brittle, callus like	Yellowish green, lamina narrow, brittle, curled, wrinkled
3.5	88.21	0.58	Yellowish green, internodes short, brittle, callus like	Yellowish green, lamina narrow, brittle, curled, wrinkled
2iP				
1.0	81.59	0.36	Yellowish green, internodes short, brittle	Yellowish green, brittle, curled
1.5	83.82	0.47	Yellowish green, internodes short, brittle	Yellowish green, brittle, curled

HS = Hyperhydric shoots; LHS = Length of hyperhydric shoots; NS = Normal shoots.

Table 3. The influence of 2iP in combination withGA_3 on hyperhydricity in small size container (90×51mm).

Hormones (mg/l)	%HS	LHS (cm)	Hyperhydricity symptoms		%NS
			Internodes	Leaf	
2iP/GA_3 1.0/2.0	70.47	0.67	Short	Curled and Wrinkled	8.72

HS = Hyperhydric shoots; LHS = Length of hyperhydric shoots; NS = Normal shoots.

Table 4. The effect of 2iP in large and small size containers on shoots regeneration from callus.

Hormone	Vessels Magnitude	% Hyperhydric Shoots	% Normal Shoot	Mean Shoot No ± SE	Mean Shoot Length (cm) ±SE
2iP					
1.0	(138×72mm)	69.32	30.68	3.4±0.36[a]	1.8±0.26[a]
1.5		75.82	24.18	2.4±0.20[b]	1.7±0.28[a]
2iP					
1.0	(90×51mm)	84.08	15.92	1.4±0.2[c]	1.4±0.2[a]
1.5		86.23	13.77	0.7±0.18[d]	1.5±0.2[a]

Mean followed by different alphabets are statistically significant at p<0.05.

Table 5. Effect of agar levels on hyperhydricity in the presence of 2iP (1.0 mg/l) in MS medium in large culture vessels.

Agar %	% Hyperhydric Shoots	% Normal Shoot	No. of Shoots ±SE	Length of Shoot (cm) ±SE
0.4	72.37	27.63	3.8±0.26[d]	2±0.21[c]
0.5	65.72	34.28	4.1±0.26[d]	3.4±0.29[b]
0.6	53.69	48.31	4.7±0.28[c]	3.5±0.29[b]
0.7	41.19	58.81	8.5±0.29[a]	5.5±0.20[a]
0.8	39.08	60.92	5.2±0.28[b]	3.5±0.20[b]

Mean followed by different alphabets are statistically significant at p<0.05.

2.2. Culture vessels and medium solidification

In order to determine the effect of culture vessels magnitude and agar concentration on induction of hyperhydricity in the shoots two types of culture vessels were used, (1) small magnitude screw cap culture vessels (90.0x51 mm) and (2) large magnitude screw cap culture vessels (138x72 mm). For solidification of medium several agar concentrations have been tested (0.4%, 0.5%, 0.6%, 0.7% and 0.8%) while pH was maintained at 5.55 to 5.56 (Table 5). One organogenic callus was placed in small culture vessels containing 15 ml of culture medium and two organogenic calluses were placed in large culture vessels containing 30 ml of culture medium. After 30 days of incubation percentage of hyperhydric and normal morphology shoots were determined (Table 4).

2.3. Optimization of medium components

Murashige and Skoog (1962) medium was modified and the organogenic callus of nodes were cultured on them as explants;

1) To confirm the effect of macroelements, concentration of ammonium nitrate (NH_4NO_3 825 mg/l) and potassium nitrate (KNO_3 950 mg/l) was decreased, and concentration of calcium chloride dehydrate $CaCl_2.2H_2O$ (880 mg/l) was increased in the standard MS medium (Table 6).
2) To confirm the existence of NH_4^+ only in the medium, KNO_3 was kept out and $CaCl_2.2H_2O$ was kept in the medium at the level of 880 mg/l. And nitrogen source was supplied in the form of NH_4NO_3 (Table 6).
3) To confirm the existence of NO_3^+ only in the medium, NH_4NO_3 was kept out and $CaCl_2.2H_2O$ was kept in the medium at the level of 880 mg/l. And nitrogen source was supplied in the form of KNO_3 (Table 6).
4) The organogenic medium contained 2 time decreased level of ammonium nitrate (412 mg/l), potassium nitrate (475 mg/l) and 2 time increased concentration of calcium chloride dehydrate (880 mg/l) of MS medium (Table 6).

All cultures were shifted into growth chamber where temperature was maintained at 25±°C. Light was provided from white fluorescent tubes in the growth chamber under a light regime of 16-h photoperiod.

2.4. Statistical analysis

All treatments were consisted of seven explants with two replicates per treatment; the experimental design

Table 6. Effect of different concentration of NH_4NO_3, KNO_3 and $CaCl_2.2H_2O$ in the medium in large size container comprising 2iP (1.0 mg/l) and agar (0.7%).

NH_4NO_3 (mg/l)	KNO_3 (mg/l)	$CaCl_2.2H_2O$ (mg/l)	% Hyperhydric Shoots	% Normal Shoot	No of Shoots ±SE
825	950	880	38.4	61.6	4.6±0.21[d]
---	950	880	35.3	64.7	5.0±0.36[d]
825	--	880	36.9	63.1	11.6±0.61[ab]
412	475	880	23.6	72.4	14.6±0.49[a]
--	475	880	31.3	60.7	9.5±0.42[bc]
412	--	880	33.7	62.3	9.0±0.36[c]
Control (Standard MS medium			46.5	53.5	5.6±0.33[cd]

Mean followed by different alphabets are statistically significant at $p<0.05$.

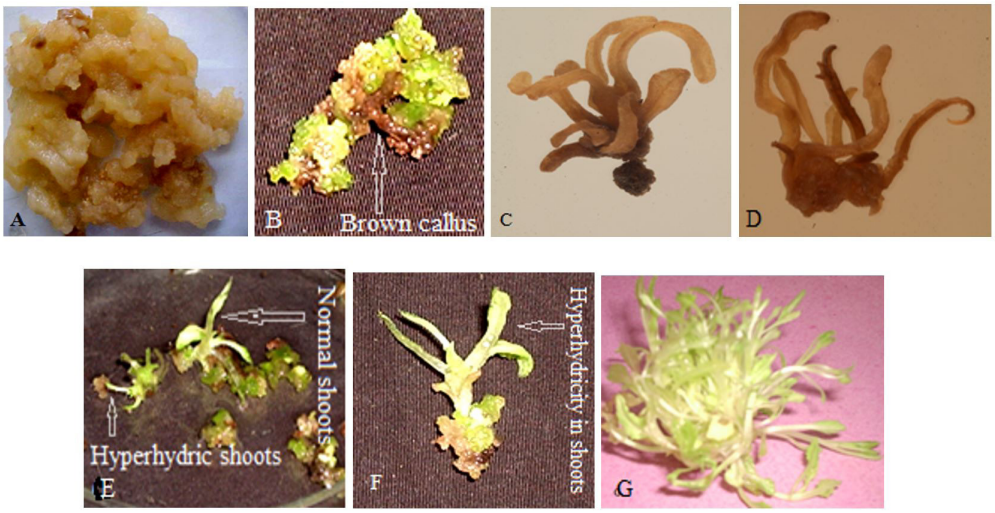

Figure 1. Adventitious shoots formation from callus of *Salvia santolinifolia*. Callus produced on MS medium, (A) callus from leaf, (B) callus from node after 25 days of culture; Hyperhydric shoots (Preserved in 70% alcohol) regenerated, (C) with 2iP (1.0 mg/l), (D) with BA (3.0 mg/l); Organogenic callus on medium, (E) Hyperhydric and normal shoot, (F) Hyperhydric shoot, (G) Normal morphology shoots.

used was completely randomize block design. Data were analyzed by applying ANOVA (SPSS statistical package software version 21.0) followed by Post hoc Tukey HSD test to check the significance difference at $p<0.05$.

3. Results

3.1. Callus development from in vitro leaf

The *In vitro* leaves of the 3rd subculture induced callus at combination of BA+NAA (4.0+0.5 mg/l) in the medium. The percent response of callus induction on leaves explants were 55 to 82% (Table 1). The calli were yellowish and friable (Figure 1A). The induced callus did not show shoots regeneration when subcultured on medium of the same composition.

3.2. Callus development from in vitro shoots

After three successive subculture of the regenerated shoot on MS medium containing BA (4.0 mg/l), small amount of callus formed at the base of shoots. The callus was whitish, granular and friable (Table 1). This callus was isolated and sub cultured to fresh medium of the similar formation. The calli first became necrotic within 3-5 days and then formed white callus from the deep-seated living cells. These white calli formed numerous small green areas after 17-20 days of cultured (Figure 1B). The green areas grew further and developed into numerous adventitious shoots which were looking glassy and fleshy and they were hyperhydric.

3.3. Subculture of hyperhydric shoots

The hyperhydric shoots and callus which have been produced on MS media supplemented with BA (4.0 and 5.0 mg/l were shifted to MS medium with reduced concentrations of BA (3.0 and 3.5 mg/l) and 2iP (1.0 and 1.5 mg/l). The original hyperhydric shoots increased in length (0.79 cm) with abnormal morphology (Table 2). While callus culture on media containing BA (3.0 and 3.5 mg/l) and 2iP (1.0 and 1.5 mg/l) replicated further and produced new green area which developed into new hyperhydric shoots. The hyperhydric shoots formed

with 2iP were yellowish green, brittle and their leaves were curled (Figure 1C) while on media supplemented with BA the hyperhydric shoots were yellowish green, brittle and their leaves were curled, wrinkled (Figure 1D). Increase percentage of hyperhydric shoots (86.72%) were observed on BA augmented media and 81.82% hyperhydric shoots were observed on 2iP containing media (Table 2). This culture condition (2iP comprised culture medium) was optimized and considered as the control. Onward experiments were planned to reduce the problem of hyperhydricity by adjusting different culture parameters.

3.4. The effect of cytokinins in combination with GA_3

Calli along with adventitious shoots (hyperhydric) were subculture on MS media, having 2iP (1.0 and 1.5 mg/l) in combination with GA3 (2.0 mg/l). All calli turned brown and then white calli emerged from necrosed callus masses which grew further and formed numerous shoots which were short, fleshy and glassy. Symptoms of these shoots were; short internodes and abnormal leaves i.e., thick, curled, wrinkled and fleshy. In spite of the presence of GA_3 in the medium shoots did not elongate more and remained small (Table 3).

3.5. Effect of vessels magnitude on hyperhydricity

The determination of this experiment was to observe the effect of culture vessels magnitude (138×72 mm &90.0×51 mm) on hyperhydricity and formation of normal morphology shoots on MS medium in the presence of 2iP (1.0 and 1.5 mg/l). After 7 days of incubation on medium in large magnitude culture vessels (138×72 mm), the callus turned brown, became necrotic and the necrotic calli then formed white callus as they did before. After 28 days of incubation it was noted that higher concentration of 2iP (1.5 mg/l) lead to numerous hyperhydric shoots along with limited numbers of normal adventitious shoots (Figure 1E) whereas lower concentration of 2iP (1.0 mg/l) lead to new crops of hyperhydric shoots with more number of normal shoots (Table 4). However, in small magnitude containers (90.0x51 mm) the callus performed the same as they did in the previous experiments (Table 2, 3) and formed reduced percentage of normal shoots. Thus, large magnitude culture vessel and cytokinin 2iP at the rate of 1.0 mg/l were optimized and used in all onward experiments.

3.6. Effect of gelling agent on hyperhydricity

Different concentrations of gelling agent were assimilated in the medium, in order to demonstrate the influence of medium solidification on hyperhydricity and regeneration of normal morphology shoots. The percentage of hyperhydricity decreased as concentrations of agar was increased progressively in the medium, highest hyperhydricity (72.37%) resulted at 0.4% agar and lowest hyperhydricity (39.08%) resulted at 0.8% agar in the medium but good quality and maximum numbers of shoots were observed at 0.7% agar (Table 5). Thus 0.7% agar was optimized and used in onward experiments.

3.7. Effect of ammonium nitrate, potassium nitrate and calcium chloride on hyperhydricity and initiation of shoot

To alleviate the problem of hyperhydricity and produce maximum number of normal morphology shoots, MS medium was modified by adding decreased concentrations of ammonium nitrate (NH_4NO_3), potassium nitrate ((KNO_3) and increased concentration of calcium chloride ($CaCl_2.2H_2O$) (of the standard level of MS) with 0.7% agar in large magnitude container (Table 6). Ammonium nitrate (NH_4NO_3) at the level of 825 mg/l, potassium nitrate (KNO_3) at the level of 950 mg/l and calcium chloride ($CaCl_2.2H_2O$) at 880 mg/l, caused the hyperhydricity to decrease drastically by 38.4% and increased the formation of normal morphology shoots by 24.37%. Only 31.3% decreased in hyperhydricity were observed when NH_4NO_3 was completely eliminated from the medium and retained KNO_3 at 475 mg/l, $CaCl_2.2H_2O$ at 880 mg/l) (Table 6). When KNO_3 was eliminated from the medium and retained other condition the same (NH_4NO_3 412 mg/l, $CaCl_2.2H_2O$ (880 mg/l), hyperhydricity decreased to 33.7%. Lowest percentage of hyperhydricity (23.6%) and highest percentage of normal morphology shoots (73.89%) (Figure 1G) was resulted in the combination of both nitrogen source (NH_4NO_3 (412 mg/l) and KNO_3 (475 mg/l) with double concentration of $CaCl_2.2H_2O$ (880 mg/l) (of the standard MS medium). Whereas maximum percentage of hyperhydric shoots was observed on the control medium ($NH_4NO_3$1650 mg/l), KNO_3 1900 mg/l and $CaCl_2.2H_2O$ (440 mg/l)) with symptom of tip necrosis of the normal shoots (Table 6).

4. Discussion

Hyperhydricity is a serious problem in the tissue culture of plants (Ziv 1991; Debergh et al., 1992) producing from stresses linked to several factors such as high humidity, high level of growth regulators, gas accumulation (Kevers et al., 2004). The hyperhydric shoots formed in this study were glassy, curled, wrinkled, water logged and translucent showing distorted growth and were very small in size, similar symptoms described by Kevers et al., (2004). The problem of hyperhydricity in micropropagation was also reported by Lebedev et al. (2018), Kadota and Niimi (2003), Ivanova et al., (2006) and Fontes et al. (1999). Cytokinins are growth hormones and its high concentrations induced hyperhydricity in the *in vitro* shoots of *S. santolinifolia*, as reported in the literature that high concentration of cytokinins causes ethylene accumulation in the cultured vessels which lead to hyperhydricity (Žd'árská et al., 2013; Liu et al., 2017). At high concentration of BA (3.5 mg/l), 85.21% explants shown hyperhydricity whereas at low concentration of BA (3.0 mg/l), 82.72% explants shown hyperhydricity along with multiple shoots formation. Liu et al. (2017) observed 94.17% hyperhydricity with Kinetin in the tissue culture of *Allium sativum*. Ivanova et al. (2006) reported that high level of exogenous cytokinin in concentration dependent manner influence hyperhidricity. A similar results stated by Vardja and Vardja, (2001), Martin et al. (2006). GA_3 has been shown to control the growth of plants by stimulating

mitotic division and cell elongation (Ali et al., 2018). In our study the addition of GA_3 in combination with BA and 2iP did not bring about elongation of hyperhydric shoots and their number also remained unaffected.

Hyperhydricity is a developmental disorder in *in vitro* regenerated shoots and various causes have been assigned to it. In the present study it appeared due to use of small culture vessels because the aeration in vessel decreased, as well as medium solidification with low agar level (0.5%) which might have resulted in producing high humidity in the culture container, increased absorption of water by the cells. High humidity of the container may also discourage wax formation on the leaves and hence impaired transpiration leading to succulence development so that the tissues appeared thickened and translucent (Collin and Edwards, 1998). With low agar concentration, more sever the hyperhydricity. Increasing agar concentration from 0.5 to 0.8% shrank hyperhydricity in our study. We discussed that low concentration of agar increases the chances of accessibility of water and humidity in the culture bottle, permitted more uptake of water, resulting hyperhydricity. It has been reported that hyperhydricity can be alleviated by increasing the concentration of agar (Casanova et al., 2008) and its reduction caused hyperhydricity (Lebedev et al., 2018). Bayraktar et al. (2020) prevented hyperhydricity by changing the gelling agent to agar-agar. The appearance of some of normal shoot in cultures which were transferred to large size culture vessels suggest that normal shoot morphogenesis requires large size containers and solid growth medium with 0.7% agar. The osmotic pressure of the medium and minimum humidity in the culture containers might have played a role in the process. The magnitude of culture vessel, ventilation, types of closure and climate parameters of the culture room all influence hyperhydration (Lai et al., 2005; Hakkaart and Versluijs, 1983). High relative humidity above the cultures accelerates the hyperhydration process (Wardle et al., 1983). The light green colour of shoots in our results may be due to chlorophyll deficiency in the regenerated shoots which have been lead to the abnormalities in the shoots of this species. Several authors identified that the light green colour of vitrified leaves may be the results of deficiency of chlorophyll (Phan and Letouze1983).

Our data in Table 6 specify that different concentrations of macro-elements (ammonium nitrate, potassium nitrate and calcium chlorite) play an important role in hyperhydricity as reported by Mazri et al. (2016) that high concentration of NH_4NO_3 in the medium resulted increased hyperhydricity. When concentrations of NH_4NO_3 and KNO_3were decreased and $CaCl_2.2H_2O$ was increased (of the standard level of MS) in the medium, the frequency of hyperhydricity decreased. The process of hyperhyricity was found reduced when the level of NH_4NO_3 and KNO_3 was reduced twice and $CaCl_2.2H_2O$ was increased twice of the standard level of MS medium. It has been reported that increased concentration of $CaCl_2.2H_2O$ in the culture medium decreased hyperhydricity in the shoots (Machado et al., 2014). Large quantities of ammonium ions have been reported to increased hyperhydricity in different species (Brand, 1993). Normal shoots with zero hyperhydricity was induced on MS medium without NH_4NO_3 (Yu et al., 2011). Our results demonstrated the increase level of calcium effect to reduced hyperhydricity. Similar result has also been reported by Machado et al. (2014).

5. Conclusions

Hyperhydricity in shoots of *S. santolinifolia* was induced by multiple factors. Hormone types and concentration, culture vessels humidity, medium solidification and modification, all had impact on hyperhydricity. The used of high level of cytokinins causes extra hyperhydricity in the shoots compare of low level. Shoots in the small culture vessels were more easily hyperhydric than those in the large culture vessels. The kind of nitrogen source affected hyperhydricity and our results advised the presence of NH_4NO_3 (412 mg/l), KNO_3 (475 mg/l), $CaCl_2.2H_2O$ (880 mg/l) (of the standard MS medium), 2iP (1.0 mg/l), agar (0.7%) essential in the medium and culture vessels magnitude (138×72mm) for decreased percentage of hyperhydricity (23.6%) and increased normal morphology shoots (73.89%).

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