

## Effects of various nitrogen sources on synchronization of tomato somatic embryogenesis during induction and realization phases

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### Abstract

Tissue culture through somatic embryogenesis is one of the methods found most useful in the plants' breeding process. A key issue to deal with during somatic evolution is its synchronization. Nitrogen has been known to play an important role here. Therefore, we evaluated the tomato explant embryogenesis cultured on B5 basal medium, subject to oxidized, reduced and organic nitrogen. Two separate experiments were conducted consisting of eight treatments with four replications each, using completely randomized design. In the experiment in which treatments were applied during the induction phase, maximum synchrony, based on relative number of torpedo embryo to all formed embryos, was obtained using nitrate as the sole nitrogen source (0.81). However, in the case in which treatments were applied during the realization phase, maximum synchrony was obtained through the combined nitrate and casein hydrolysate (0.51). Furthermore, in both experiments the highest number of somatic embryos was obtained in the standard B5 medium (91.58 in the first experiment and 59.19 in the second experiment).

**Keywords:** Ammonium; Casein hydrolysate; *In vitro*; Nitrate; Tomato

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### Introduction

Tissue culture studies clarified that most plant cells have the potential of forming somatic embryos through a process named somatic embryogenesis (Bhojwani and Dantu 2013). Currently, plant biotechnology, genetic transformation and other similar techniques depend on the plant regeneration by means of somatic embryogenesis (Kaur and Kapoor 2016). Several influential factors involve in the success of this process. Among these factors, plant species, low ammonium content and cell cluster size have been involved (Fujimura 2014). Using response surface methodology to study the effect of MS salts on *in vitro* propagation of red raspberries (*Rubus idaeus*

L.), Poothong and Reed (2014) found nitrogen as the most important nutrient. Also, previous studies have proven the effectiveness of amino acids and casein hydrolysate on date palm somatic embryogenesis (Al-Khayri 2011; Khierallah and Hussein 2013). In addition, it was shown that the relative proportion of oxidized versus reduced nitrogen played an important role in the success of pumpkin suspension culture and somatic embryogenesis (Mihaljević *et al.* 2011; Pěnčíket *et al.* 2015). Poothong and Reed (2016) also reported that *Rubus idaeus* var. *Nepalensis* regeneration was maximized in the mediums containing minimum proportion of ammonium to nitrate. Different effects of various forms of nitrogen on

synchronization of somatic embryogenesis were also reported (Mashayekhi-Nezamabadi 2000). One of the most notable challenges is asynchrony of embryogenesis which is found to be the major cause of low conversion frequency of somatic embryos (Kumar *et al.* 2014).

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable crop, belonging to Solanaceae family which comes second in popularity around the world (FAO 2011). Therefore, establishing a proper technique for its mass production is important. Despite previous works, there are no conclusive reports on the effects of nitrogen separately on induction, realization or synchronization of somatic embryogenesis. Since the modes of nitrogen effects on formation and development of somatic embryogenesis still remains controversial, the aim of this investigation was the assessment of synchronization of tomato somatic embryogenesis due to participation of nitrogen in the induction and realization phases of somatic embryogenesis.

### Materials and Methods

Experiments on induction and realization phase were conducted separately. Sterile seeds were cultured in glass jars containing 25 ml solid MS basal medium (Murashige and Skoog 1962). Liquid cultures were initiated directly from primary explants by isolating hypocotyl segments of two weeks old tomato seedlings. Explants obtained from sterile seedlings were cultured in 150 ml of liquid B5 (Gamborg *et al.* 1968) medium. In Experiment 1 cultures were inoculated

in a B5 medium already modified with different forms of nitrogen (Table 1). In Experiment 2 a standard B5 basal medium was used for the induction phase of somatic embryogenesis. In both experiments induction medium was supplemented with 1 mg/L 2,4-D considering a 30-day time duration for the induction. Prior to transferring explants into realization medium, explants were washed out by hormone free medium three times for periods of 5, 10 and 15 minutes (Mashayekhi-Nezamabadi 2000), then transferred into hormone free realization medium. For Experiment 1, B5 basal medium nominated for realization medium, while for Experiment 2 the modified B5 medium was used which was subjected to different nitrogenous compounds (Table 1). Modification of nitrogen type alters amounts and proportions of  $K^+$  and  $SO_4^-$  ions in the culture medium. Therefore, equal molar ratio of  $K_2SO_4$  was added to the mediums to compensate the omitted  $KNO_3$  or  $(NH_4)_2SO_4$ . The pH level of all mediums was adjusted to 5.7 before autoclaving. Cultures were incubated in  $25 \pm 2^\circ C$ , under constant illumination. Oxygen supplied for cultures by means of auxophyton with revolution of 1 RPM. Number of somatic embryos were counted under binocular. The data reported on the number of somatic embryos for each treatment, refer to six explants per replication.

Synchronization of somatic embryogenesis was indexed based on the number of torpedo embryos in relation to number of all formed embryos in each culture unit.

Table 1. Concentration of various forms of nitrogen in the induction medium of Experiment 1 and realization medium of Experiment 2 (mg/L).

Medium	NO <sub>3</sub> <sup>-</sup>	NH <sub>4</sub> <sup>+</sup>	Casein hydrolysate
1	415.6	28.4	32.5
2	476	0	0
3	0	476	0
4	0	0	476
5	233	238	0
6	238	0	238
7	0	238	238
8	0	0	0

## Results and Discussion

Successful somatic embryogenesis of tomato in B5 culture medium was observed in this study (Figure 1). In both experiments, the highest frequency of somatic embryos was obtained with B5 medium (Table 2) which seems to be due to presence of well-adjusted amounts of all three forms of nitrogen. A high frequency embryogenesis was also obtained with the experiment with casein hydrolysate as sole nitrogen source in the induction medium (Table 2). Previous reports have also shown the efficiency of casein hydrolysate on somatic embryogenesis of date palm (Al-Khayri 2011; Khierallah and Hussein 2013) and Japanese larch (*Larix leptolepis*) (Kim and Moon 2007). However, a few number of somatic embryos were obtained in the realization phase by casein hydrolysate as the only nitrogen source. Therefore, different mechanisms may be considered for casein hydrolysate involvement during induction and realization. In the experiment that induction medium contained ammonium and standard B5 medium was used for realization phase, just a small number of somatic embryos formed. In the experiments that induction medium was standard B5 medium and the realization phase contained

ammonium as sole nitrogen, the minimum number of somatic embryos were observed (Table 2).

Considering the potential role of ammonium on somatic embryogenesis of pumpkin, Mihaljevic' *et al.* (2011), attributed the failure of somatic embryogenesis to the lack of inorganic nitrogen. Furthermore, it was asserted that ammonium-grown bromeliad plant showed a drastic repression of indole acetic acid (IAA) (Endres *et al.* 2002). Accordingly, low frequency of somatic embryogenesis in such medium can be also attributed to the lower endogenous auxin level in response to high ammonium concentration. Frequency of somatic embryos which was induced in the medium contained both ammonium and casein hydrolysate was more than the medium contained only ammonium as the sole nitrogen in the induction phase (Table 2). Previous reports had also refuted the efficiency of high concentrations of ammonium (> 40 mM) (Mihaljevic' *et al.* 2011) and enhancing effect of casein hydrolysate on somatic embryogenesis (Kim and Moon 2007; Al-Khayri 2011; Khierallah and Hussein 2013). Nitrate treatment, nitrate with casein hydrolysate and nitrate with ammonium brought about acceptable frequencies of somatic embryos in both

experiments. Although the frequency of somatic embryos by the combined application of nitrate and casein hydrolysate was acceptable in realization phase, their frequency was more considerable during the induction of somatic embryogenesis. Combination of ammonium with nitrate or casein hydrolysate in the culture media was superior to ammonium as a single source of nitrogen. This could be due to either smaller amounts of

ammonium in the combined treatments relative to single sources of nitrogen in the culture media (476 ppm with ammonium as a single source of nitrogen) or greater efficiency of casein hydrolysate and nitrate relative to ammonium.

The ratio of torpedo-stage embryos relative to all embryos was defined as the synchronization index. Nitrate-grown explants brought about maximum synchronization value (Table 3).

Table 2. Comparison of means for number of somatic embryos affected by different forms of nitrogen.

Medium	Experiment (1)	Experiment (2)
1	91.78a <sup>†</sup>	59.19a
2	56.18ab	50.92ab
3	10c	44.91d
4	88.47a	21.47c
5	68.43ab	35.79abc
6	86.33a	40.34ab
7	46.83b	30.79bc
8	††	††

<sup>†</sup>Different letters denote significant differences among different treatments for number of somatic embryos. Means followed by the same letter are not significantly different at 5% probability level by LSD test.

<sup>††</sup>There were no or a few embryos in the related treatment, thus no analysis was done in this regard.

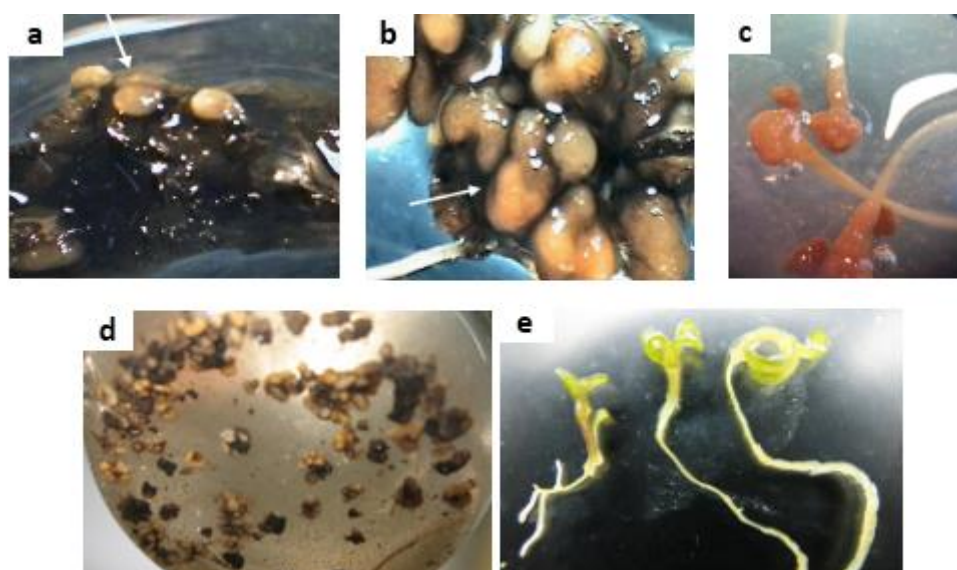


Figure 1. Tomato somatic embryogenesis in B5 culture medium; a) globular embryo regenerated from tomato hypocotyl; b) early torpedo embryo regenerated directly from tomato hypocotyl; c) embryoid structure formed in B5 medium containing nitrate as sole nitrogenous source; d) regenerated embryos in the culture tubes; e) regenerated plantlet from tomato somatic embryos (Bar= 100 micron).

Table 3. Comparison of treatment means for ratio of torpedo stage to all embryos.

Medium	Experiment (1)	Experiment (2)
1	0.68 <sup>ab†</sup>	0.28 <sup>b<sup>c</sup></sup>
2	0.81 <sup>a</sup>	0.32 <sup>b</sup>
3	0 <sup>d</sup>	0 <sup>d</sup>
4	0.14 <sup>dc</sup>	0 <sup>d</sup>
5	0.32 <sup>c</sup>	0.34 <sup>b</sup>
6	0.38 <sup>bc</sup>	0.51 <sup>a</sup>
7	0.34 <sup>c</sup>	0.15 <sup>dc</sup>
8	††	††

†Different letters in each column denote significant differences among different treatments. Means followed by the same letter are not significantly different at 5% probability level by LSD test.

††There were no or a few embryos in the related treatment, thus no analysis was done in this regard.

Studying the *Arabidopsis thaliana* nitrate transporter NRT1.6, Almagro *et al.* (2008) also found that nitrate was important for early embryo development. In presence of nitrate, cells keep dividing actively which is prerequisite for embryogenesis (Trigiano and Gray 2004). Embryoid structures was also observed in the medium containing nitrate as the sole nitrogen (Figure 1a), which can be due to high frequency of biological-active auxin in the presence of nitrate transporter (Almagro *et al.* 2008) and consequent participation of auxin in formation of embryoid which was reported about barley microspore (Cai *et al.* 1992).

In experiment with the induction in the standard B5 medium, the maximum synchrony was obtained in the presence of combined nitrate and casein hydrolysate (Table 3). As mentioned before, nitrate is known to reinforce cell proliferation (Trigiano and Gray 2004) and casein hydrolysate is recognized as a factor for development, cell wall synthesis and final quiescence (Mashayekhi-Nezamabadi 2000). Moreover, in the presence of amino acid substrates, low molecular weight amide conjugation of auxin with amino acids (IAA-ASP,

IAA-GLU) is likely (Ludwig-Müller 2011). This conjugation represses endogenous auxin activity (Zimmerman 1993) which is a condition favored for realization of somatic embryos (Böttcher *et al.* 2012). Therefore, it could be concluded that casein hydrolysate in a mixture of various amino acids hinders IAA biological activity and promotes somatic embryo development. It seems that cells which had already been divided recurrently in the presence of nitrate were differentiated in the presence of casein hydrolysate.

The present study showed that various forms of nitrogen affect induction and realization phases of tomato somatic embryogenesis differently which impacts their synchronization. Up to now, a little attention has been paid to the effect of nitrogen on synchronization of somatic embryogenesis. Therefore, current findings in this study may be useful towards a closer approach to understanding the basis of synchronization in plant somatic embryogenesis. Assessing the pattern of expression of genes involved in the induction of embryogenesis and nitrogen metabolism is recommended for further research.

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**Conflict of interest**

The authors declare that they have no competing interests.

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## اثر منابع مختلف نیتروژن روی هم‌زمان سازی جنین زایی رویشی گوجه فرنگی در طی مراحل القا و ظهور جنین

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### چکیده

کشت بافت از طریق جنین زایشی رویشی یکی از روش‌هایی است که بیشتر در روند اصلاح گیاهان کاربرد دارد. یک مسئله کلیدی جنین زایی رویشی، هم‌زمان سازی آن است. مشخص شده است که نیتروژن در این مورد نقش مهمی ایفا می‌کند. بنابراین، آزمایشی برای ارزیابی جنین زایی رویشی گوجه فرنگی در محیط پایه B5، تحت تاثیر نیتروژن اکسیدی، احیایی و آلی قرار گرفت. دو آزمایش جداگانه شامل هشت تیمار با چهار تکرار هر کدام با استفاده از طرح کاملاً تصادفی انجام شد. در آزمایشی که تیمارها در طول مرحله القا، اعمال شده بودند، حداکثر هم‌زمان سازی براساس تعداد نسبی جنین اژدری شکل به تمام جنین‌های تشکیل دهنده جنین، با استفاده از نیترات به عنوان تنها منبع نیتروژن (۰/۸۱) به دست آمد. در مقابل، زمانی که در طول مرحله ظهور جنین تیمارها اعمال شدند، حداکثر هم‌زمان سازی با استفاده از ترکیب نیترات و هیدرولیزات کارژین (۰/۵۱) به دست آمد. علاوه بر این، در هر دو آزمایش بیشترین تعداد جنین رویشی در محیط استاندارد B5 (۹۱/۵۸) در اولین آزمایش و ۵۹/۱۹ در آزمایش دوم) ثبت شد.

**واژه‌های کلیدی:** آمونیم؛ درون شیشه ای؛ کارژین هیدرولیزات؛ گوجه فرنگی؛ نیترات