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RESEARCH ARTICLE

IN VITRO EFFECT OF NITROGEN IN LARGE SCALEPROPAGATION OF THREE CULTIVAR OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

Anshu Rani, Varun Kumar, Priyanka and *Sandeep Kumar

Department of Biotechnology, NIET, NIMS University, Jaipur-303121, Rajasthan, India

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ABSTRACT

The effect of nitrogen was investigated on the micro propagation of three cultivars (Angoorlata, K5-7, A3ad-8) of Tomato *(Lycopersicon esculentum* MILL.) to overcome the challenges related to its cultivation. Sterilized seeds of three cultivars were transferred on hormone free medium and used stem as explants for culture initiation. The stemexplant was cultured on MS medium fortified with BAP (0.5-5.0mg/l) along with Kn(0.5-5.0mg/l), maximum shoot multiplication were recorded on BAP (3mg/l) in cultivars. Six weeks old multiple shoots were transferred on modified MS medium containing Ca $(NO_3)_2.4H_2O$ (50-300 N mg/l), KNO_3 (50-300 Nmg/l) with BAP (2.5mg/l) were observed. The highest shoot multiple on MS medium with Ca(NO3)2.4H2O (50-300 N mg/l), KNO_3 (50-300 N mg/l) with BAP (2.5mg/l) in Angoorlata, K5-7 and A3ad-8. Moreover, 80% of them were able to re-grow when sub-cultured on same media. The multiple shoots were transferred on MS medium with or without nitrogen IAA(0.5-2.0 mg/l), IBA(0.5-2.0 mg/l) and α-NAA(0.5-2.0 mg/l) for rooting find out to be best and observed IAA (1mg/l)gives maximum best rooting for all cultivars. With calcium nitrate and potassium nitrate were recorded. Healthy rooted plantlets were transferred on hardening mixture of Farmyard manure: Garden soil. Plantlets were shifted in mist house for acclimatization. 87% plants survival rate in Angoorlata and K5-7 (82%) and 78% plant survival rate in A3ad-8 cultivar were recorded seven weeks old plants.

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INTRODUCTION

Tomato is one of the most widely grown vegetables in the world. It is one of the most important protective foods as it possesses appreciable quantities of vitamins, minerals and sometimes rightly referred to as poor man's orange (Devi *et al.* 2008). Tomato belongs to *"Solanaceae"* family and botanical name of Tomato is "*Lycopersicon esculentum* MILL.". Tomato is a genus of about 7500 species of herbs and shrub in the nightshade family. It is a perennial, annual plant and native to the tropical region in Europe and Asia. Tomato originated from South America. It was spread to the Caribbean, Philippines, Asia and Europe by the Spanish. Plant tissue culture techniques are being used nowadays to overcome the challenges related to the propagation of plants. This technique provides a large number of plantlets in very short span of time. The plantlets produced are homogeneous with the parents and also provides seasonal independence for the production of plants (Bhatia *et al.,* 2004; Tiwari *et al.,* 2013; Anshu *et al.,* 2016). Nitrogen is one of the major components in MS media (which is most commonly used plant tissue culture media) and significantly influences the growth and morphogenesis of *In vitro* plant culture (Gamborg *et al.,* 1968; Kumar et al., 2003). In tissue culture medium nitrate, ammonium salt, amino acids, and complex organic products supply nitrogen. The amount and form of nitrogen in the basal medium have significant effects on the rate of cell growth, differentiation and cell tot potency (Mengel *et al.,* 2013, Yadav *et al.,* 2013, Kumar *et al.,* 2017). Keeping all the above-mentioned points in view the present investigation was undertaken with a motive to understand the role of nitrogen sources in micropropagation of tomato cultivars which would be further helpful in opening doors in the understanding of its plant physiology regarding its growth, differentiation and development with respect to the research area. Three cultivar of tomatoes using bimodal explant; the most regeneration variety could be then efficiently micro propagated for commercial purpose and molecular studies.

Corresponding author: Sandeep Kumar,
Department of Biotechnology, NIET, NIMS University, Jaipur-303121, Rajasthan, India.

METHODS AND METHODOLOGY

Plant material

Seeds of tomato cultivars for the study will obtain from Chandra Shekhar Azad University of Agriculture Science and Technology, Kanpur.

Seed surface sterilization and seed germination

The seeds were immerse in distilled water and treated with Bavistin 1% solution for 45 minutes followed by thoroughly rinsing with tap water. Two drop of Tweeen-20 per 100ml were add to the seeds and soaked thoroughly for 15 minutes and thoroughly rinsed with sterile distilled water for 4 to 5 times. The seeds were taken into laminar air flow cabinet and treated with 70% ethyl alcohol for 30 sec followed by treatment with (0.1%) $HgCl_2$ for 5 minutes and then wash for 4 to 5 times with double distilled water. Sterilized seeds were inoculate in test tubes containing MS medium (Murashige and Skoog 1962) without hormones and transferred in the dark room for germination of all cultivars. Germinated seedling served as explants source for tissue cultured experiments.

Culture media and inoculation

The stem of explant were inoculated on to sterilized (autoclaved at 1.1 kg/cm^{3}for 20 minutes at121°C) semisolid (3% sucrose, 0.8 % agar with pH 5.8 ± 0.2,) basal MS medium (Murashige and Skoog 1962) supplemented with various concentrations and combinations of different auxins α-NAA (0.5-5.0mg/l) and IAA (0.5 – 5.0mg/l) and cytokinins, BAP (0.5-5.0mg/l), kinetin (0.5-5.0mg/l) alone and in combination BAP (0.5-5.0mg/l), kinetin (0.5-3.0mg/l) was used. MS medium were used for making a cost effective and regenerative protocol for shoot multiplication. Cultures were incubated at 25 ± 1°C with a photoperiod of 16 hours at 2000-2500 lux of cool white fluorescent light. Shoots were initiated after 8 – 12 days of inoculation and then sub-cultured regularly on fresh medium at five weeks interval.

Shoot regeneration medium

Binodal explant was cultured on MS medium supplemented with different combinations and permutation of BAP (0.5-5.0mg/l), Kn (0.5-5.0mg/l) and α-NAA (0.5-5.0mg/l) for multiple shoot regeneration. The optimization of media with hormones was then followed by test in nine modified MS media which differed in their nitrogen sources i.e., $Ca(NO_3)_2.4H_2O$ (50-300 N/l), KNO_3 (50-300 N/l). After six weeks of inoculation, the regenerated shoots were then studied morphologically and effects of nitrogen on growth and differentiation were evaluated.

Rooting medium

After optimization of nitrogen concentrations elongated micro shoots were excised from culture tube and transferred to MS medium supplemented with different concentrations and combination of IAA (0.5-3.0mg/l), IBA (0.5-3.0mg/l)and α-NAA (0.5-3.0mg/l) individually for rooting.

Acclimatization and transfer to field

Plantlets with well-developed roots were transferred to plastic trays for hardening which contained autoclaved garden soil and farmyard manure.

Acclimatization was standardized for its time period, relative humidity and temperature conditions before the plantlets were transplanted in to the soil in field condition. New study was initiated after 5-6 days of inoculation. Three replications were tested for each treatment and repeated three times after six weeks data on number of shoots/explant, number of roots/explant, shoot length, root length were recorded.

RESULTS

Present study was undertaken to establish a responsible protocol for mass scale micro propagation of local elite cultivars of tomato. Morphological parameters such as explant response, number of shoots and length of shoots (in cm) were estimated for the nodal explant in micropropagation of three cultivars of Tomato (*Lycopersicon esculentum* MILL.). The present study was undertaken with BAP and Kn, BAP proves to be having way more prudent impact on shoot induction likewise as on proliferation as compared to Kinetinin three varieties tomato. These results were comparable previous finding where BAP was reported best hormone for shoot formation in tomato (Rani *et al.,* 2016, Kumar *et al.,* 2017).

Effect of auxins and cytokinines on shoots proliferation

In vitro sterilized seeds were transferred on hormones free MS medium. After 3-4 days, seeds were start germinating on MS medium in dark chamber. 80% seed viability was observed in all cultivars of tomato (Fig. 1).Seven weeks old seedling has 6cm height with 5-6 nodes seedling. Binodal segment explant was used for the induction of shoots on various combinations of auxin and cytokinins (Fig. 2). BAP 3.0 mg/l alone showed maximum on shoot induction with maximum average number of shoots 6.4 while highest shoot length 6.69cmin a variety Angoorlata where in K5-7, the average number of shoots were recorded6.8 with average shoots length up to 6.3cm and in A3ad-8 variety have average number of shoots and length of shoots was 9.4 and 6.2cm respectively. Kinetin (0.5-5mg/l) was used for the same parameters and Kinetin (3mg/l) alone proved to be and having average number of shoots 4.35 and average length of shoots 3.47cm found in Angoorlata variety, where average number of shoots and length of shoots 4.2 and 3.5cm respectively found in K5-7 variety.

The kinetin concentration give result in variety A3ad-8 and showed average number of shoots 4.3 and length of shoots 3.5cm.The combination of two cytokinins BAP (0.5-5mg/l) and Kn (0.5-5mg/l) was taken to study the effect. The combination of BAP (2mg/l) and Kn (2mg/l) was proved to be best combination in Angoorlata, K5-7 and A3ad-8. The combination of BAP and Kn gives average number of shoots and average length of shoots 6.31, 6.3, 6.2 and 4.2cm, 4.1cm, 4.0cm respectively in all three varieties of tomato (Graph 1, 2, 3). Further BAP (0.5-5 mg/l) was used with different auxins IAA (1-5 mg/l) and α- NAA (1-5 mg/l). BAP (2mg/l) with α-NAA (3mg/l) was recorded to be best in all cultivars of tomato, having average number of shoots 4.43, 4.6, 4.2 and average length of shoots 3.59cm, 3.6cm, 3.4cm respectively. Combination of BAP with α-NAA showed better result rather than BAP with IAA for initiation of multiple shoot proliferation in Angoorlata, K5-7 and A3ad-8. After six weeks old culture were transferred

Effect of Nitrogen on shoot proliferation

The effect of Calcium nitrate and Potassium nitrate was tested on the process of multiplication in micro propagation of

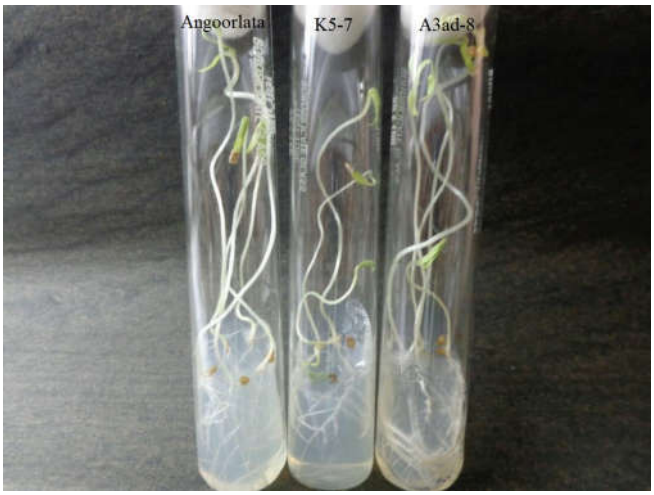


Fig. 1. *In vitro* seed germination on hormones free MS medium (4 weeks old)

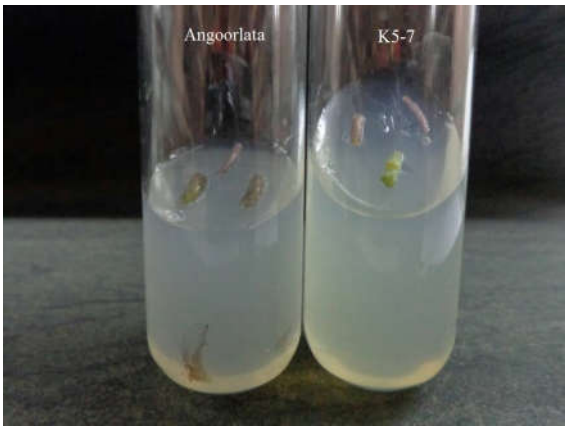
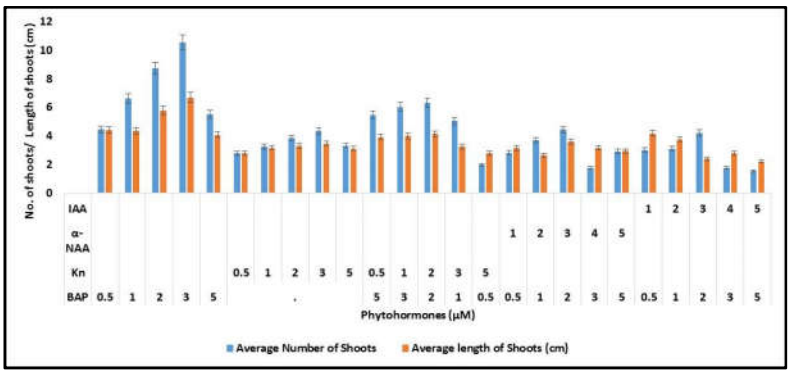
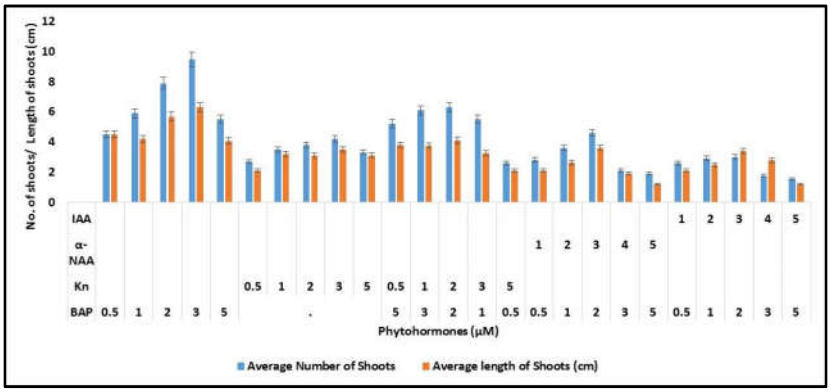


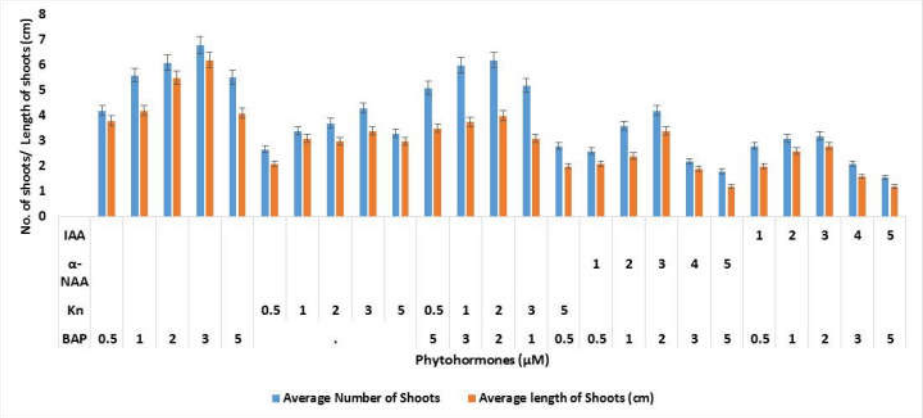
Fig. 2: Induction of shoots on MS media with BAP



Graph 1. Effect of auxins and cytokinines along and in different combinations over Angoorlata cultivar



Graph 2. Effect of Auxins and Cytokinines along and in different combinations over K5-7 cultivar



Graph 3. Effect of auxins and cytokinines along and in different combinations over A3ad-8 cultivar

Fig. 3. Multiplication of shoots on MS media with BAP+ Nitrogen

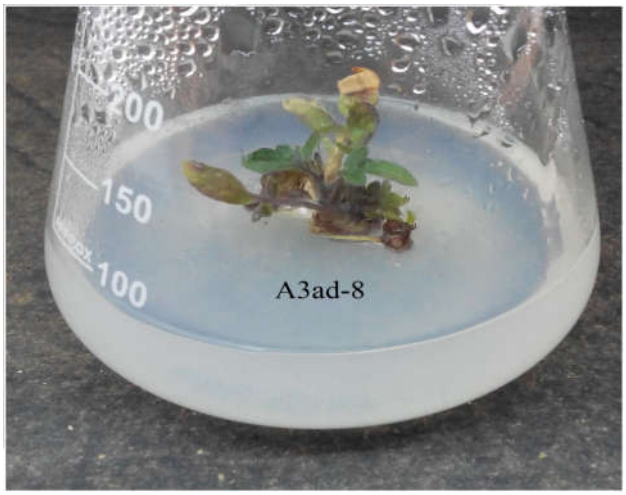
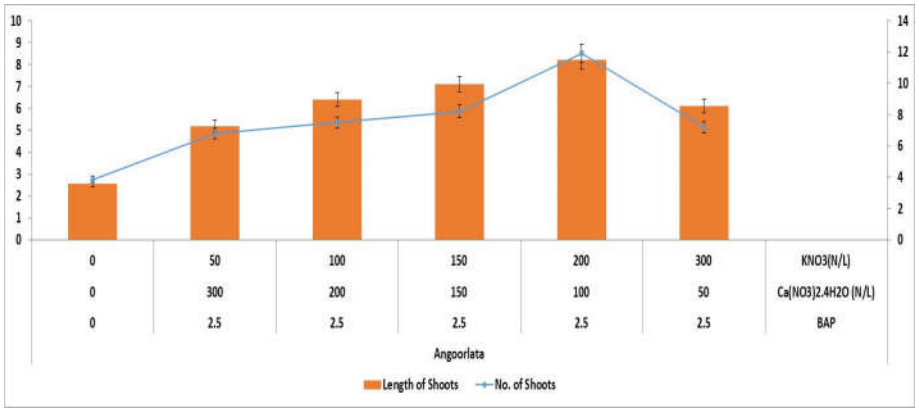
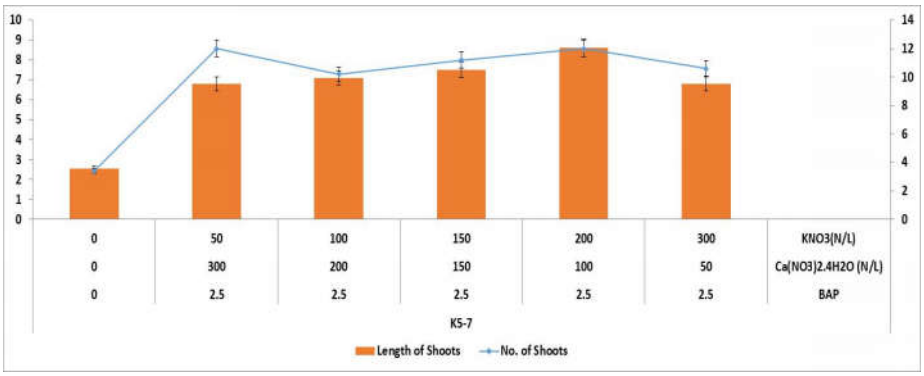


Fig. 4: Multiplication of shoots on MS media with BAP+ Nitrogen

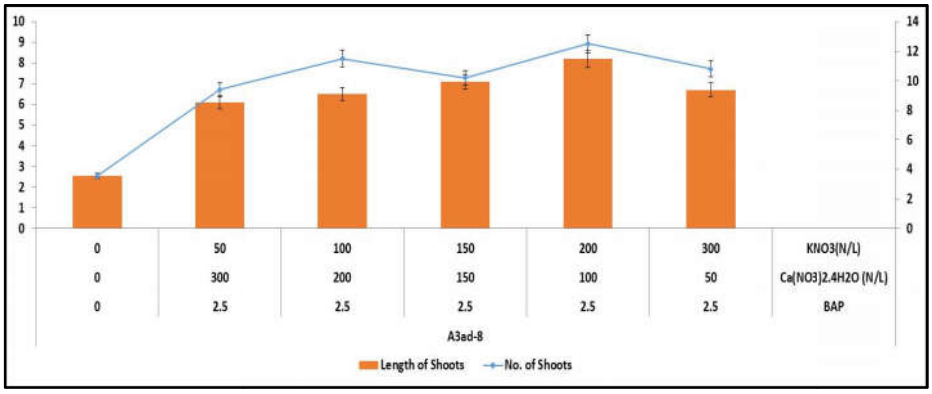


Graph 4. Effect of nitrogen for shoot induction and multiplication in Angoorlata cultivar.

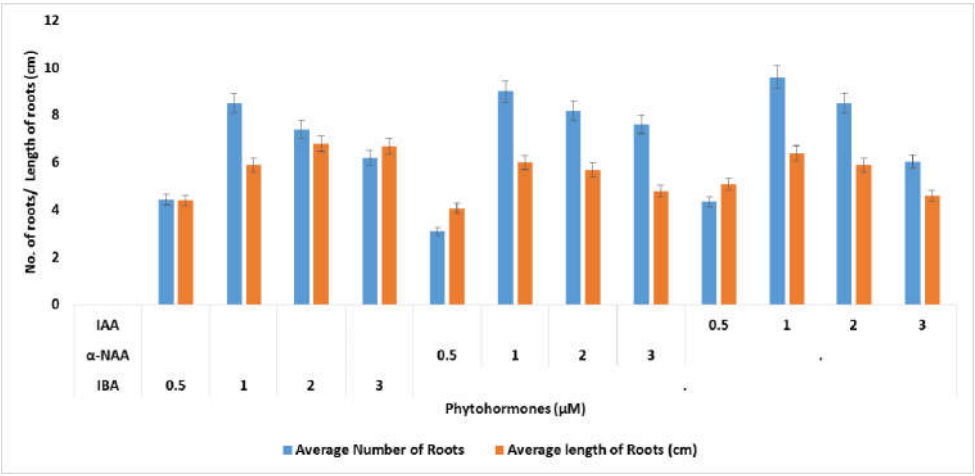
tomato cultivars. Ca $(NO_3)_2$. $4H_2O$(50-300 N mg/l) and KNO_3(50-300 N mg/l) were added with BAP (2.5mg/l) and observations were recorded at six weeks. Up to six week of culture, the length and the number of shoots were found to be increased. In the six weeks culture observations the maximum number of shoots and shoot length was produced on MS medium supplemented with the combination of $Ca(NO_3)_2.4H_2O$ (100 N mg/l)+ KNO_3(200 N mg/l) gives number of shoots 11.9 and average of length of shoots 8.2 cm in angoorlata, K5-7 cultivar it give number of shoots 12 and length of shoots 8.6 cm, A3ad-8 cultivar gives average number of shoots 12.5 and average length of shoots 8.2 cm(Graph 4, 5, 6) (Fig. 3,4).The best type and concentration of nitrogen source added as media supplement were resulted out. It is also evident from this study that the nitrogen sources have a significant impact over the process of multiplication.



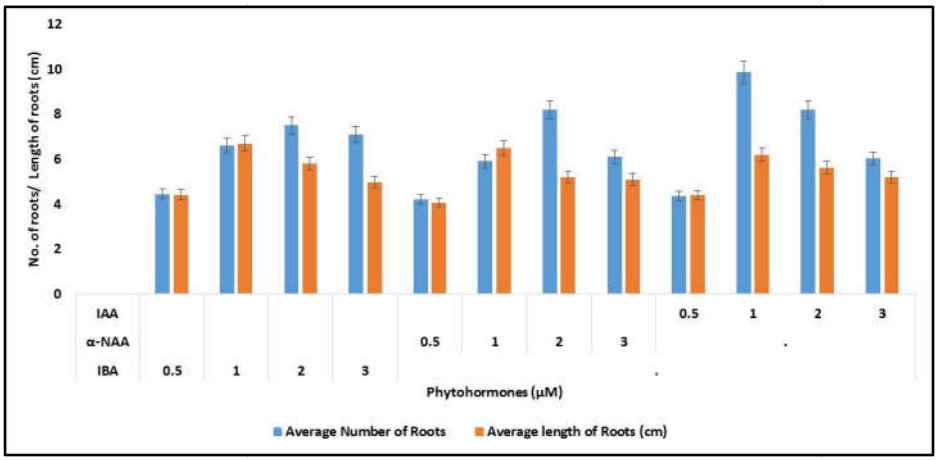
Graph 5. Effect of nitrogen for shoot induction and multiplication in K5-7 cultivar



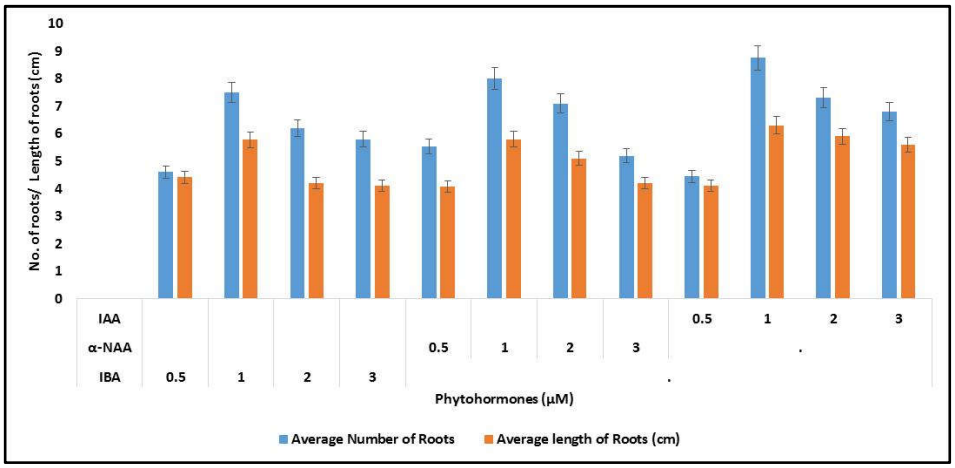
Graph 6. Effect of nitrogen for shoot induction and multiplication in A3ad-8 cultivar



Graph 7. Effect of different auxins for the root induction in Agoorlata cultivar



Graph 8. Effect of different auxins for the root induction in K5-7 cultivar



Graph 9. Effect of different auxins for the root induction in A3ad-8 cultivar

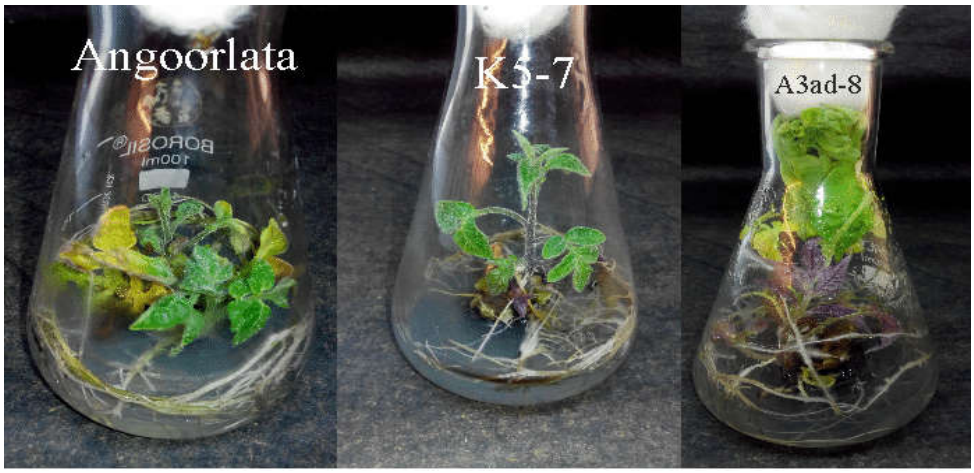
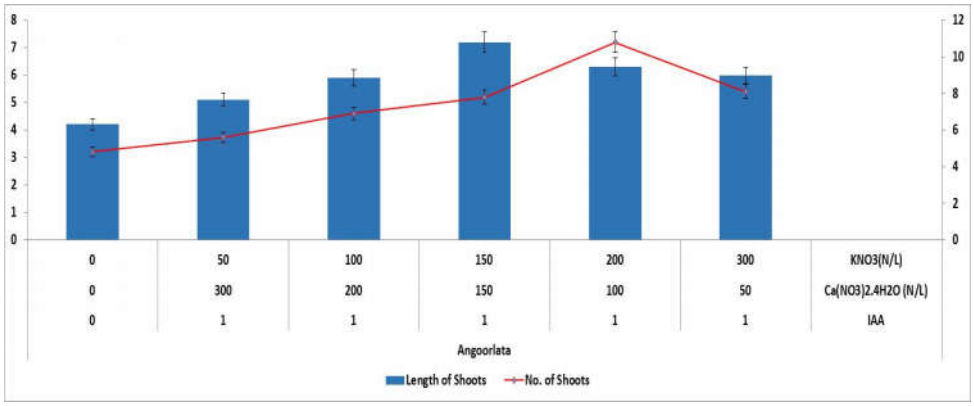


Fig. 5. *In vitro* rooting on MS media with Nitrogen+ IAA (Seven weeks old plantlets)



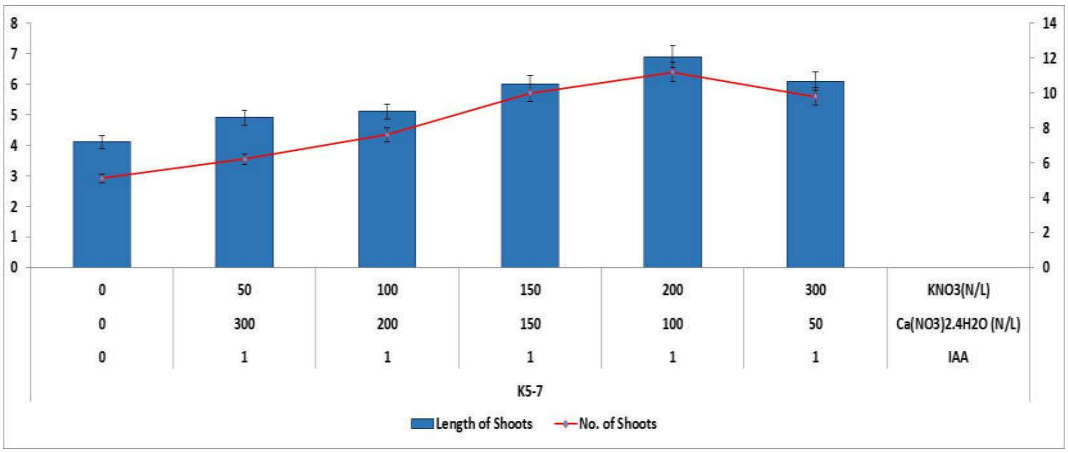
Graph 10: Effect of nitrogen for root multiplication in Angoorlata cultivar

Effect of Auxins on Rooting

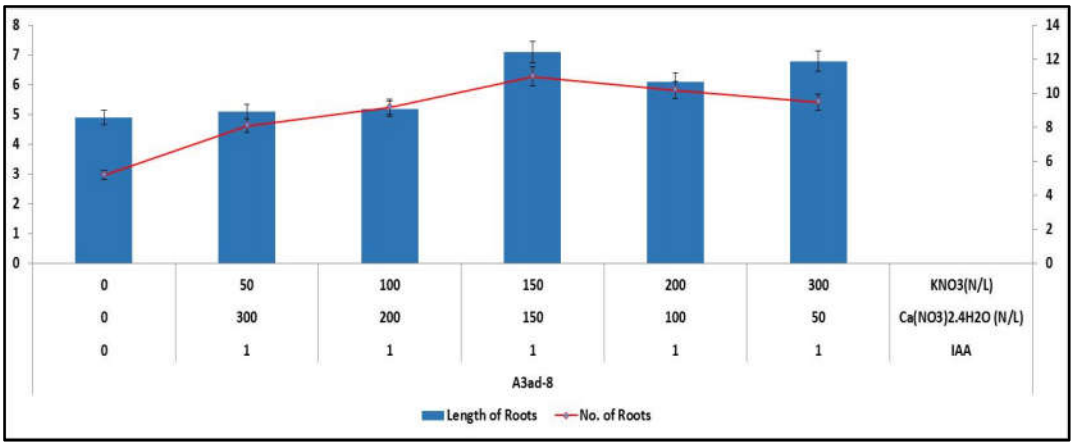
The efficacy of different auxins (IAA, IBA, α-NAA) with varying concentration (0.5-3.0mg/l) was tested for the root initiation of multiplied shoots on MS media. Two parameters, the number of roots per shoots and average length of root. The maximum number of roots was observed on K5-7 (9.86) followed by Angoorlata (8.6) and A3ad-8 (8.75) on MS media with 1mg/l IAA. The root induction and multiplication gradually decrease with increasing concentration of IAA. In K5-7 cultivars, maximum number of roots (8.2) on MS media with (2mg/l) α-NAA and length of roots on 1mg/l α-NAA. MS media supplemented with 1mg/l α-NAA were recorded maximum number of roots and length in Angoorlata and A3ad-8 (Graph 7, 8, 9).With 1mg/l IAA was proved the best auxin for root induction and it was reported best in the previous finding over tomato (Rani *et al.,* 2016).

Effect of Nitrogen on Rooting

Healthy multiple shoots were subcultured on MS media with same nitrogen concentration as shoot multiplication along with IAA (1.0mg/l) and observed maximum number of roots (11.2) and length of roots (6.9cm)in K5-7 on calcium nitrate 100 Nmg/l, potassium nitrate 200 N mg/l. (Graph 11). In Angoorlata cultivar, six weeks maximum number of roots (10.8) was recorded with concentration of 100N mg/l, 200N mg/l and increased length (7.2cm) of roots was recorded with



Graph 11. Effect of nitrogen for root multiplication in K5-7 cultivar



Graph 12. Effect of nitrogen for root multiplication in A3ad-8 cultivar

150 N mg/l, 150 N mg/l of $Ca(NO_3)_24H_2O$ and KNO_3 (Graph 10). In A3ad-8 cultivar, maximum number of roots and length of roots were observed same concentration as Angoorlata (Graph 12). The use of nitrogen combination to develop healthy root system was also advocated that root formation much better in media devoid of nitrate nitrogen (Fig. 5). The fully developed plantlets were removed from the test tube and transplant to the sterilized garden soil and farm yard manure in the pot. One hundred plantlets were transferred for all cultivars. Among the different genotypes highest percentage (87%) of plantlets in the case of Angoorlata was survived in garden soil and farmyard manure and establish as healthy plants. Lowest percentage (78%) of hardening efficiency was recorded in case of A3ad-8 followed by K5-7 (82%).

DISCUSSION

Enhanced axillary branching was utilized for micropropagation of tomato cultivars. *In vitro* culture response in tomato is dependent on the age of explants. In the present investigation the axillary bud induction was achieved from preexisting quiescent meristem from the seeding of shootings of tomato showed not good response through: experiments were also conducted to achieve bud induction process. Cytokinins are the major class of growth regulators responsible for *In vitro* shoots induction (Skoog, 1971). BAP and Kn used, BAP showed thebest response in the proliferation of axillary buds (Kumar *et al.,* 2013, Yadav *et al.,* 2013). BAP (3mg/l) was found best for axillary bud induction and multiplication. Addition of little amount of auxins α-NAA and IAA was found to have the stimulatory effect on bud induction.

The use of inorganic nitrogen for plant development was in alignment with Ramage and Williams (2002) results from that study showed a synergistic effect of nitrate on shoot regeneration. Calcium nitrate and Potassium nitrate were added in combination and results were recorded at six weeks. These results were supported by Villamor (2010) the texture of shoots was green. The minimum number and length of shoots was observed when media was not supplemented with nitrogen source. These results showed a co-ordination with Sen and Batra (2011), they reported that there was no morphological changes and growth when *phyllanthu samarus* explants were cultured on MS medium devoid of nitrogen sources. It was found that media with high nitrogen content was much better for plant *In vitro* regeneration. The various combinations of nitrogen on the formation of roots on MS medium along with IAA and α-NAA were recorded (Yadav *et al.,* 2013, Priyanka *et al.,* 2016). The cultivars showed variation in their response to growth and a cultivars dependent response to a combination of nitrogen, auxins and cytokinine for the multiplication of K5-7, Angoorlata and A3ad-8 cultivars. This protocol meets the objectives of the study and provide solid basis for the commercial mass production of the studied cultivars through *In vitro* microprapagation techniques.

Conclusion

The present investigation reports an efficient and easy to handle protocol for micro propagation through axillary nodal segment for tomato cultivars. In content, the best explant selection the maximum 5 fold shooting and 85% rooting was found in bimodal segment explant.

The best growth regulator, nitrate nitrogen selection experiment. BAP for shooting and IAA for rooting with nitrogen superior in all cultivars of tomato. The different concentrations of nitrogen exerted the variable effect on the formation of shoots, roots. The concentration of nitrogen also affected the growth of shoots and roots.

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