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Full Length Research Paper

Enhanced regeneration in explants of tomato (*Lycopersicon esculentum* L.) with the treatment of coconut water

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A standardized protocol was developed to enhance the *in vitro* regeneration efficiency of five varieties of tomato from hypocotyls and leaf disc with the involvement of coconut water. Different concentrations of IAA and kinetin were used alone and in combination with 12% of coconut water. Significant differences for regeneration, time taken to regenerate and number of leaf primordial were observed for different treatments, type of explant, use of coconut water and also among varieties. Higher regeneration was obtained in Avinash followed by Roma and Rio Grande. Maximum regeneration 95.75% was obtained with 0.5 mgL^{-1} of IAA, 1.5 mgL^{-1} Kinetin along with 12% coconut water in Avinash and hypocotyl was proved to be better for regeneration than leaf discs. The inclusion of coconut water in the media significantly reduced the number of days taken for callus induction leading to regeneration, as compared to media without coconut water. Significant increase in number of shoot primordial was observed in hypocotyls as well as in leaf disc derived calli with the addition of coconut water. Plants obtained were transferred to the glass house in small pots of compost and higher frequency of survival was observed from plantlets obtained with the addition of coconut water.

Key words: Tomato, coconut water, regeneration, days to maturity, shoot primordia.

INTRODUCTION

Environmental stresses are major factors limiting the growth and production of crops. The development and improvement of stress tolerance of crops are primary targets for plant molecular and genetic breeding (Yuasa et al., 2007). Tomato is a major vegetable crop that has achieved tremendous popularity over the last century throughout the World. Its cultivation is limited by various factors including fungi, bacteria, viruses and nematodes. Jones et al. (1991) presented major diseases of tomato caused by fungi, bacteria, viruses and various nematodes. Development of protocols for *in vitro* selection can provide new advances for the production of stress tolerant cultivars. Somatic embryogenesis in tomato is still at its infancy and efficient procedures for large scale production via somatic embryogenesis are yet to be developed. Several primitive species of the genus *Lycopersicon*, especially *Lycopersicon peruvianum*, *Lycopersicon hirsutum* and *Lycopersicon glandulosum*, represent an important source of genes, conferring resistance to various diseases and pests of cultivated tomatoes (Lukyanenko, 1991). However, introduction of those genes to commercial cultivars of *Lycopersicon esculentum* by conventional breeding techniques often encounters serious difficulties due to the high incompatibility barriers to hybridization (Kaul, 1991). To overcome these problems, certain modern approaches of gene manipulation might be required.

Genetic engineering techniques are being used in tomato improvement programs to fight these challenges.

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Abbreviations: CW, Coconut water; **IAA,** indole acetic acid; **BAP,** 6-benzylaminopurine.

Table 1. List and composition of media used for regeneration with 3% sugar and pH adjusted to 5.7 - 5.8.

Media	Composition
RM_1	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 0.5 mgL^{-1}
RM_2	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 1mgL^{-1}
RM_3	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 1.5 mgL^{-1}
RM_4	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 2 mgL^{-1}
RM_5	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 2.5 mgL^{-1}
RM_6	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 0.5 mgL^{-1} + 12% CW
RM_7	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 1mgL^{-1} + 12% CW
RM_8	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 1.5 mgL^{-1} + 12% CW
RM_9	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 2 mgL^{-1} + 12% CW
RM_{10}	MS salts and vitamins + IAA 0.5 mgL^{-1} + Kinetin 2.5 mgL^{-1} + 12% CW

A genetic engineering program requires: delivery of appropriate DNA into the cell; integration of the introduced DNA into the chromosome; for stable transformation; selection of transformed cells (promoters and markers) and *in vitro* regeneration of the transformed cells. A good *in vitro* regeneration system is essential for an effective genetic engineering system that seeks to exploit genetically transformed plants for commercial applications. Prolonged period of calli to initiate shoot primordia and consequently the delayed formation of whole plants from *in vitro* cultures of tomato reduced the efficiency of the further plant development in various ways. The commercial use of plant tissue culture primarily involves the production of a large number of plants with minimum input expenses. The main factors that ultimately influenced the commercial propagation of plants *in vitro* are: the screening of genotypes, the physical environment and the chemical media for *in vitro* culture. The development of a cost effective and efficient protocol for mass propagation of high quality tomato seedlings via tissue culture could help to reduce the price per seedling. A good *in vitro* plant regeneration system may also assist in further improvement of the commercially important cultivars for disease resistance via genetic engineering.

Growth regulators are also one of the most important components of the *in vitro* culture media. Since the pioneering work of that zeatin has been widely accepted as the only cytokinin capable of inducing satisfactory growth in tomato explants (Jabeen et al., 2009) but number of shoot primordia produced is not addressed before in tomato *in vitro* culture. However, due to its high cost, there is also a generalized opinion that an alternative replacement should be achieved for use in commercial micro propagation protocols. Coconut water (CW) and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation (Peixe et al., 2007). CW had been reported in other important orchids micropropagation, such as monopodial orchid hybrid *Aranda deborah* with 20% CW (Lakshmanan et al., 1995) and *Arachnis labrosa* (Temjensangba and Deb, 2005), monocots as well such as in maize (Baskaran et al., 2006), sugarcane (Desai et al., 2004). Because of stimulating effects on cell division, CW has been used in this study to determine its effects on callus induction and regeneration. It can then be utilized to enhance the transformation frequency, because less time can increase the production and survival of transgenic plants, which is our ultimate goal. Therefore, the present communication describes a simple and efficient procedure for enhanced regeneration of tomato plants by the treatment with CW *in vitro*.

MATERIALS AND METHODS

Seed collection, preparation and germination

Seeds of tomato (*L. esculentum* L.) var. Rio Grande, Roma, Pusa Ruby, Pant Bahr and Avinash were obtained from Horticulture Research Institute, National Agriculture Research Centre, (Islamabad, Pakistan). Seeds were washed with tap water for about 10 min before surface sterilization with 0.8% (v/v) "Clorox" bleach (sodium hypochlorite) for 10 min, followed by three rinses (5 min each) with sterile distilled water under aseptic conditions in a laminar flow cabinet. Surface sterilized seeds of tomato were then planted in MS medium (Murashige and Skoog, 1962) and placed in a growth chamber under white fluorescent light (600 µmol m^{-2} s^{-1}; 16 h light/8 h dark) at 25°C and 70% relative humidity for germination. Leaves and hypocotyls of the five varieties were collected from 3-week-old seedlings.

Explant preparation, callus induction and regeneration

From the 2 - 3 week-old *in vitro* seedlings, hypocotyls and leaf discs of about 1 cm in length were utilized as explants to assess callus induction and regeneration on five different media combinations with and without 12.0% CW as shown in (Table 1). Media formulations consisted of different concentrations of IAA and Kinetin either in combination with 12% CW or without inclusion of CW along with other adjuvants having gelrite as the solidifying agent. The experiment was conducted in four replicates with 100 explants in each replication for both types of explants. Data were recorded on the number of calli induced, number of shoot primordial formed and

Table 2. Analysis of variance for effect of CW on regeneration in tomato varieties.

K value	Source	D.F	S.S	M.S	F value	Probability
1	Replication	2	8.368	4.184	1.9652	0.1428
2	Genotypes (A)	4	9894.887	2473.722	1161.6953	0.0000
4	Explant (B)	1	43372.405	43372.405	20372.196	0.0000
6	AB	4	1192.186	298.047	139.9669	0.0000
8	Treatment (C)	9	63627.729	7069.7477	3320.6894	0.0000
10	AC	36	5100.978	141.694	66.5415	0.0000
12	BC	9	4207.961	467.551	219.5688	0.0000
14	ABC	36	4351.149	120.865	56.7601	0.0000
-15	Error	198	421.623	2.129		
	Total	299	132177.285			

number of days to maturity for *in vitro* regeneration.

Rooting of shoots and transfer of plantlets to soil

As the tomato shoots began to regenerate from calli, they were transferred to rooting media supplemented either with IAA 0.1 mgL^{-1} and the number of shoots that produced roots was recorded after a three-week incubation period. All media contained 3% sucrose with pH adjusted at 5.76 and were solidified with 4 gL^{-1} of gelrite. Rooted plants derived from hypocotyls and leaf discs after one week of root formation were shifted to small pots of compost in the glass house. They were covered with a polyethylene bag for 10 - 12 days and watered in 4 - 5 days intervals. After two weeks, they were shifted to big pots. Ten plants obtained from seeds were also grown in the green house. The plants were harvested after maturity. Frequency of survived plants was recorded.

Statistical analysis

Data were recorded on the frequency of calli induction, morphology of the calli, viability of embryogenic calli, regeneration frequency and other numerical parameters accordingly. All experiments were laid out in randomized complete block design. Each treatment was replicated thrice and ten test tubes in the case of callus induction and eight flasks in the case of regeneration were used for replication. The data collected was analyzed by using MSTAT-C statistical software and the means were compared by least significance difference test using MSTAT-C (Steel and Torrie, 1984), with significance being recorded at $p \leq 0.01$.

RESULTS

Effect of IAA, Kinetin and CW on regeneration percentage of tomato varieties

Leaf disc and hypocotyls from 3 weeks old *in vitro* seedlings were used for callus induction on five media without (RM_1-RM_5) and with (RM_6-RM_{10}) CW (Table1). Analysis of variance for the response of regeneration under various media combinations in various tomato genotypes is shown in Table 2. Statistically, significant differences for genotypes, explants, treatments and for all kinds of their interactions that is, genotype × explant, genotype × treatment, explant × treatment and genotype × explant × treatment were recorded. Regeneration was achieved on all the MS media combinations supplemented with IAA and Kinetin (RM_1-RM_5 –ive CW; RM_6-RM_{10} +ive CW). Maximum regeneration percentage in both types of explants that is, leaf disc and hypocotyls was recorded in RM_8 medium (+ive CW) and RM_3 (-ive CW) medium in all genotypes (Table 3). Roma gave the highest percentage of regeneration with leaf disc explants in RM_8 medium (60.60%) followed by Avinash (59.17%), Rio Grande (58.02%), Pant Bahr (40.58%) and Pusa Ruby (36.1%) (Table 3; Figure 1). However, comparatively higher percentage of regeneration was observed in hypocotyls explants at the same medium (RM_8) in all the genotypes that is, in Avinash (95.65%) followed by Rio Grande (91.43%), Pusa Ruby (89.33%), Roma (87.53%), and Pant Bahr (59.9%) (Table 3). Regeneration medium without CW, maximum regeneration was observed at RM_3 medium in Roma (50.2%), Rio Grande (48.25%), Pusa Ruby (43.47%), Avinash (39.40%) and Pant Bahr (31.67%) with leaf discs (Table 3). Similarly, for hypocotyls in RM_3 medium without CW; maximum regeneration percentage was determined in Avinash (89.57%) followed by Rio Grande (85.3%), Roma (79.5%), Pusa Ruby (69.5%) and Pant Bahr (60.33%) (Table 3). RM_9 medium (+ive CW) ranked 3rd in leaf disc explants derived regeneration percentage with highest in Avinash (49.57%), followed by Roma (40.87%), Rio Grande (38.77%), Pusa Ruby (26.37%) and in Pant Bahr (30.58%). In the same medium for hypocotyls, maximum regeneration percentage was established in Avinash (75.27%), Roma (71.00%), Rio Grande (60.72%), Pusa Ruby (68.60%) and Pant Bahr (51.00%). In case of RM_4 (-ive CW) medium for leaf disc explants, highest regeneration percentage was observed in Avinash (50.93%) followed by Rio Grande (39.3%), Roma (34.68%), Pusa Ruby (28.42%) and Pant Bahr (20.65%). While for the hypocotyls; Rio Grande gave maximum percentage of regeneration (75.25%), followed by 69.48, 58.58, 53.1 and 49.78% in Roma, Avinash, Pusa Ruby and Pant Bahr, respectively in RM_4 media (Table 3). Other media

Table 3. Comparison of hypocotyls and leaf disc derived regeneration of five tomato varieties on MS regeneration media supplemented with or without CW with different media combinations as mentioned in Table 1.

Media	Hypocotyls						Leaf discs					
	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean
RM_1	25.5 ^{H}	25.75 ^{G}	25.5 ^{F}	20.0 ^{F}	30.08 ^{I}	25.37 ^{I}	10.5 ^{F}	9.75 ^{H}	10.0 ^{F}	11.5 ^{F}	27.0 ^{E}	13.75 ^{H}
RM_2	65.50 ^{D}	61.25 ^{D}	41.0 ^{D}	50.25 ^{B}	60.25 ^{E}	55.65 ^{E}	35.5 ^{D}	29.25 ^{E}	29.0 ^{C}	21.25 ^{D}	39.17 ^{C}	30.83 ^{E}
RM_3	85.3 ^{B}	79.5 ^{B}	69.5 ^{B}	60.33 ^{A}	89.57 ^{B}	76.85 ^{B}	48.25 ^{B}	50.20 ^{B}	43.47 ^{A}	31.67 ^{B}	39.40 ^{C}	41.40 ^{B}
RM_4	75.25 ^{C}	69.48 ^{C}	53.1 ^{C}	49.78 ^{B}	58.58 ^{E}	61.24 ^{D}	39.30 ^{C}	34.68 ^{D}	28.42 ^{C}	20.65 ^{D}	50.93 ^{B}	34.80 ^{D}
RM_5	52.80 ^{F}	39.60 ^{E}	34.53^{E}	25.25 ^{E}	40.97 ^{H}	38.63 ^{G}	19.45 ^{E}	24.57 ^{F}	20.90 ^{D}	16.98 ^{E}	22.10 ^{F}	20.80 ^{G}
RM_6	30.42 ^{G}	29.33 ^{F}	35.30^{E}	29.42^{D}	44.77 ^{G}	33.85 ^{H}	8.33 ^{F}	15.63 ^{G}	18.65^{DE}	26.37 ^{C}	30.53 ^{D}	19.90 ^{G}
RM_7	76.25 ^{C}	70.47 ^{C}	55.32^{C}	50.5 ^{B}	80.03 ^{C}	66.51 ^{C}	41.57 ^{C}	39.45 ^{C}	35.23 ^{B}	30.52 ^{B}	40.30 ^{C}	37.41 ^{C}
RM_8	91.43 ^{A}	87.53 ^{A}	89.33^{A}	59.9 ^{A}	95.65^{A}	84.77 ^{A}	58.02 ^{A}	60.60 ^{A}	36.10 ^{B}	40.5 8 ^{A}	59.17 ^{A}	51.09 ^{A}
RM_9	60.72 ^{E}	71.00 ^{C}	68.60^{B}	51.00 ^{B}	75.27 ^{D}	65.32 ^{C}	38.77 ^{C}	40.87 ^{C}	26.37 ^{C}	30.58 ^{B}	49.57 ^{B}	37.23 ^{C}
RM_{10}	59.17 ^{E}	40.88 ^{E}	39.33^{D}	39.93 ^{C}	48.68 ^{F}	45.60 ^{F}	19.60 ^{E}	29.47 ^{E}	17.60 ^{E}	30.78 ^{B}	30.75 ^{D}	25.64 ^{F}
Mean	62.24 ^{A}	57.48 ^{B}	51.15^{C}	43.64^{D}	62.39 ^{A}		32.03 ^{B}	33.45 ^{B}	26.57 ^{C}	25.99 ^{C}	38.89 ^{A}	

Means not followed by the same letter within a group are significantly different at LSD = 0.01.

were less responsive for regeneration in both types of explants for all the genotypes. On the average irrespective of the explants nature, RM_8 (+ive CW) and RM_3 (-ive CW) were found the most responsive media for *in vitro* regeneration (Table 4). However, these two media were statistically different to each other. RM_9 (+ive CW) and RM_4 (-ive CW) gave the 3rd and 4th highest regeneration percentage for the genotypes under study. From all the varieties tested in this study, Rio Grande, Avinash and Roma were found the most responsive varieties. However, regeneration percentage was significantly different among these genotypes. Pusa Ruby and Pant Bahr were less responsive varieties for the *in vitro* regeneration.

Effect of CW on days to maturity

The analysis of variance for days to maturity in tomato varieties on different media compositions is given in Table 5. Statistically, significant variations were recorded for genotypes, explants, treatments as well as for various interactions that is, genotype × explant, genotype × treatment, explant × treatment and genotype × explant × treatment.

A marked influence of CW in the media on the calli was observed for number of days to regenerate in *in vitro* environment (Table 6). Lowest number of days of maturity for hypocotyls was found in Avinash (15.17, 19.55, 19.38 days) in the RM_7, RM_8 and RM_{10} with the inclusion of CW (Table 6) as compared to the respective treatments that is, RM_2, RM_3 and RM_5 without CW. Rio Grande ranked 2nd for the response of CW followed by Pusa Ruby, Roma and Pant Bahr as far as the regeneration days from hypocotyls were considered. For hypocotyls explants minimum number of days was taken by the RM_6 followed by RM_7, RM_8, RM_9 and RM_{10} media and were statistically similar among each other. Generally, lower number of days to maturity was taken by the leaf disc as compared to the hypocotyls. All the varieties gave similar response to the CW in the media at the respective treatments for leaf disc explants and non significant difference for days to regeneration was recorded among the treatments having CW. For leaf disc minimum number of days was taken by the RM_9 followed by RM_8, RM_6, RM_{10} and RM_6 media and was statistically similar among each other. By considering the mean number of days to maturity irrespective of the explant source, minimum days was taken by the RM_7, followed by RM_8, RM_9, RM_{10} and RM_6 media, statistically were similar among themselves. Maximum days to maturity were taken by the treatment RM_5, followed by RM_1, RM_2, RM_4 and RM_3 media (Table 7). Figures 1 and 2 demonstrate the different stages of regeneration in all varieties tested.

Effect of CW on number of shoot primordial

Analysis of variance for number of shoot primordia

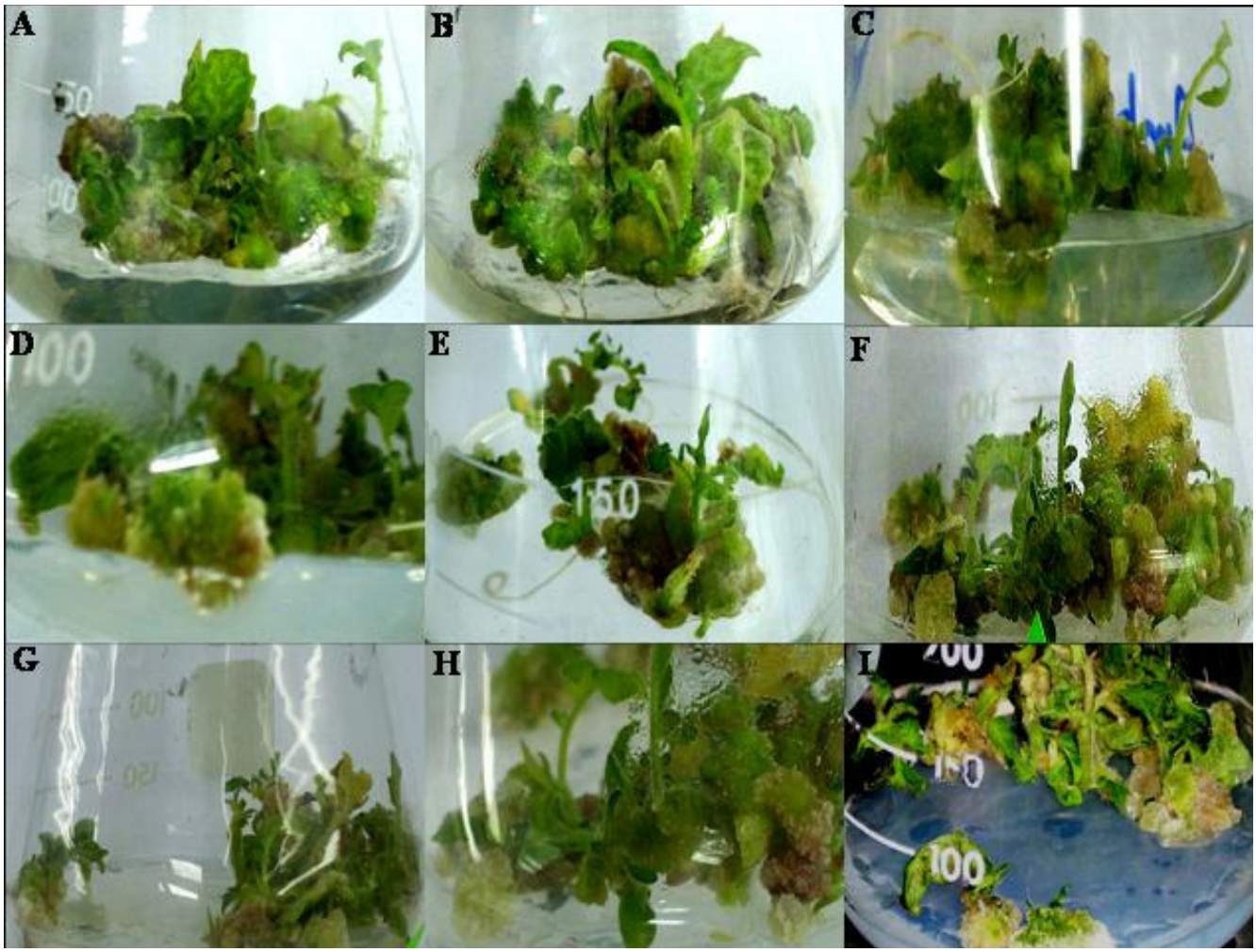

Figure 1. Shoot proliferation in different tomato varieties tested with CW. (A and B) Rio Grande callus proliferation with morphogenesis in to shoots of leaf discs on media containing coconut after 7 days of culturing, (C) callus with multiple shoots on RM_8 medium 10 days after culturing, (D) shoots multiplication from leaf disc derived callus after 12 days on RM_8, (E) Roma hypocotyl derived shoot proliferation with multiple shoots on RM_7, (F) leaf disc derived shoot proliferation with multiple shoots on RM_7; 12 days after culturing, (G) Avinash hypocotyl derived shoot proliferation with shoot on RM_7; 18 days after culturing, (H) multiple embroids coming out of leaf disc derived callus to form shoots and (I) callus with shoot proliferation on RM_8 in Avinash.

Table 4. Effect of shoot regeneration of five tomato varieties on MS regeneration media with different media combinations as mentioned in Table 1.

Treatment	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean
RM_1	18.00 ^{G}	17.75 ^{I}	17.75 ^{G}	15.75 ^{G}	28.54 ^{H}	19.56 ^{I}
RM_2	50.50 ^{D}	45.25 ^{E}	35.00 ^{E}	35.75 ^{D}	49.71 ^{E}	43.24 ^{E}
RM_3	66.79 ^{B}	64.85 ^{B}	56.48 ^{B}	45.5 ^{B}	64.48 ^{B}	59.62 ^{B}
RM_4	57.28 ^{C}	52.08 ^{D}	40.76 ^{D}	35.22 ^{D}	54.76 ^{D}	48.02 ^{D}
RM_5	36.13 ^{F}	32.08 ^{G}	27.72 ^{F}	21.12 ^{F}	31.53 ^{G}	29.72 ^{G}
RM_6	19.38 ^{G}	22.48 ^{H}	26.98 ^{F}	27.89 ^{E}	37.65 ^{F}	26.88 ^{H}
RM_7	58.91 ^{C}	54.96 ^{C}	45.28 ^{C}	40.51 ^{C}	60.17 ^{C}	51.96 ^{C}
RM_8	75.22 ^{A}	74.07 ^{A}	62.72 ^{A}	50.24 ^{A}	77.41 ^{A}	67.93 ^{A}
RM_9	49.74 ^{D}	55.93 ^{C}	47.48 ^{C}	40.79 ^{C}	62.42 ^{BC}	51.27 ^{C}
RM_{10}	39.38 ^{E}	35.17 ^{F}	28.47 ^{F}	35.36 ^{D}	39.72 ^{F}	35.62 ^{F}
Mean	47.13 ^{B}	45.46 ^{C}	38.86 ^{D}	34.81 ^{C}	50.64 ^{A}	

Means not followed by the same letter within a group are significantly different at LSD = 0.01.

Table 5. Analysis of variance for days to maturity of different tomato varieties.

K value	Source	D.F	S.S	M.S	F value	Probability
1	Replication	2	17.558	8.779	2.48345	0.08605
2	Genotypes (A)	4	248.570	62.143	17.5812	0.0000
4	Explant (B)	1	3787.981	3787.981	1071.5646	0.0000
6	AB	4	79.583	19.896	5.6289	0.0003
8	Treatment (C)	9	32597.391	3621.9323	1024.5919	0.0000
10	AC	36	1172.959	32.582	9.2181	0.0000
12	BC	9	77.914	8.657	2.4492	0.0115
14	ABC	36	250.003	6.945	1.9647	0.0019
-15	Error	198	699.850	3.535		
	Total	299	38931.809			

D.F = Degree of freedom; S.S = sum of squares; M.S = mean sum of squares.

Table 6. Comparison of days to maturity of hypocotyls and leaf disc in tomato varieties on MS regeneration media supplemented with or without CW along with 3% sucrose with IAA 0.5 mg L-1, kinetin, 4 g L-1 of gelrite.

Media	Hypocotyls						Leaf discs					
	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean
RM_1	40.5 ^{AB}	48.8 ^{A}	49.3 ^{A}	39.53 ^{B}	42.73 ^{BC}	44.1 ^{AB}	33.47 ^{B}	40.5 ^{A}	41.27 ^{A}	32.3 ^{BC}	35.63 ^{A}	36.64 ^{A}
RM_2	40.57 ^{AB}	47.2 ^{A}	49.6 ^{A}	41.34 ^{B}	41.52 ^{C}	44.0 ^{AB}	31.00 ^{B}	39.9 ^{A}	39.2 ^{AB}	35.7 ^{AB}	31.28 ^{B}	35.4 ^{AB}
RM_3	39.27 ^{B}	42.6 ^{B}	45.0 ^{B}	40.154 ^{B}	39.62 ^{C}	41.40 ^{C}	32.57 ^{B}	35.1 ^{BC}	35.6 ^{BC}	31.13 ^{C}	36.67 ^{A}	34.30 ^{B}
RM_4	43.33 ^{AB}	42.1 ^{B}	40.8 ^{C}	39.62 ^{B}	46.27 ^{AB}	42.3 ^{BC}	35.00 ^{B}	35.5 ^{C}	31.27 ^{D}	35.7 ^{AB}	36.60 ^{A}	34.21 ^{B}
RM_5	44.00 ^{A}	47.2 ^{A}	44.3 ^{BC}	49.87 ^{A}	47.93 ^{A}	46.08 ^{A}	39.27 ^{A}	37.8 ^{AB}	34.6 ^{CD}	37.82 ^{A}	38.67 ^{A}	37.63 ^{A}
RM_6	19.45 ^{C}	21.2 ^{D}	25.3 ^{D}	24.58 ^{CD}	24.0 ^{D}	22.91 ^{D}	15.67 ^{C}	16.5 ^{D}	15.72 ^{C}	15.43^{D}	19.40 ^{C}	16.55 ^{C}
RM_7	19.83 ^{C}	25.7 ^{C}	23.9 ^{D}	20.52 ^{D}	15.17 ^{F}	21.02 ^{D}	14.58 ^{C}	15.7 ^{D}	18.27 ^{E}	14.85 ^{D}	14.87 ^{C}	15.66 ^{C}
RM_8	20.53 ^{C}	22.4 ^{CD}	20.0 ^{E}	25.46 ^{C}	19.55 ^{E}	21.60 ^{D}	16.47 ^{C}	14.3 ^{D}	15.37 ^{E}	16.32 ^{D}	14.27 ^{C}	15.34 ^{C}
RM_9	21.43 ^{C}	22.3 ^{CD}	21.6 ^{DE}	25.27 ^{C}	20.42 ^{D}	22.21 ^{D}	16.23 ^{C}	14.5 ^{D}	14.17 ^{E}	15.43 ^{D}	15.27 ^{CD}	15.11 ^{C}
RM_{10}	21.33 ^{C}	23.2 ^{CD}	23.5 ^{DE}	25 ^{C}	19.38 ^{E}	22.55 ^{G}	15.50 ^{C}	15.0 ^{D}	17.55 ^{E}	17.47 ^{D}	16.30 ^{CD}	16.37 ^{C}
Mean	30.92 ^{B}	34.3 ^{A}	34.3 ^{A}	32.9 ^{AB}	31.66 ^{B}		24.99 ^{A}	26.23 ^{A}	26.31 ^{A}	25.22 ^{A}	25.90 ^{A}	

Means not followed by the same letter within a group are significantly different at LSD = 0.01.

8. There was found highly significant variations for genotypes, explants, treatments as well as for various interactions that is, genotype × explant, geno-type × treatment, explant × treatment and genotype × explant × treatment (Table 8).

Addition of CW enhanced the number of shoot primordia development against the respective treatments without CW with both types of explant source (Table 9).

Table 7. Days to maturity of five tomato varieties on MS regeneration media supplemented with or without CW along with 3% sucrose with IAA 0.5 mg L^{-1}, kinetin, 4 g L^{-1} of gelrite.

Treatment	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean
RM_1	36.98 ^{B}	44.65 ^{A}	45.30 ^{A}	35.94 ^{B}	39.18 ^{BC}	40.41 ^{AB}
RM_2	35.83 ^{B}	43.58 ^{A}	44.43 ^{A}	38.52 ^{B}	36.40 ^{C}	39.75 ^{BC}
RM_3	35.92 ^{B}	39.05 ^{B}	40.31 ^{B}	35.83 ^{B}	38.14 ^{C}	37.85 ^{D}
RM_4	38.67 ^{B}	37.47 ^{B}	36.02 ^{C}	37.67 ^{B}	41.43 ^{AB}	38.25 ^{CD}
RM_5	41.63 ^{A}	42.53 ^{A}	39.47 ^{B}	42.34 ^{A}	43.30 ^{A}	41.86 ^{A}
RM_6	17.56 ^{C}	18.88 ^{C}	20.51 ^{DE}	20.01 ^{CD}	21.70 ^{D}	19.73 ^{E}
RM_7	17.21 ^{C}	20.70 ^{C}	21.08 ^{D}	17.68 ^{D}	15.02 ^{E}	18.34 ^{E}
RM_8	18.50 ^{C}	18.36 ^{C}	17.68 ^{E}	20.89 ^{C}	16.91 ^{E}	18.47 ^{E}
RM_9	18.83 ^{C}	18.37 ^{C}	17.91 ^{E}	20.35 ^{CD}	17.84 ^{E}	18.66 ^{E}
RM_{10}	18.42 ^{C}	19.15 ^{C}	20.54 ^{DE}	21.23 ^{C}	17.84 ^{E}	19.44 ^{E}
Mean	27.95 ^{BC}	30.25 ^{A}	30.33 ^{A}	29.05 ^{AB}	28.78 ^{AB}	

Means not followed by the same letter within a group are significantly different at LSD = 0.01.

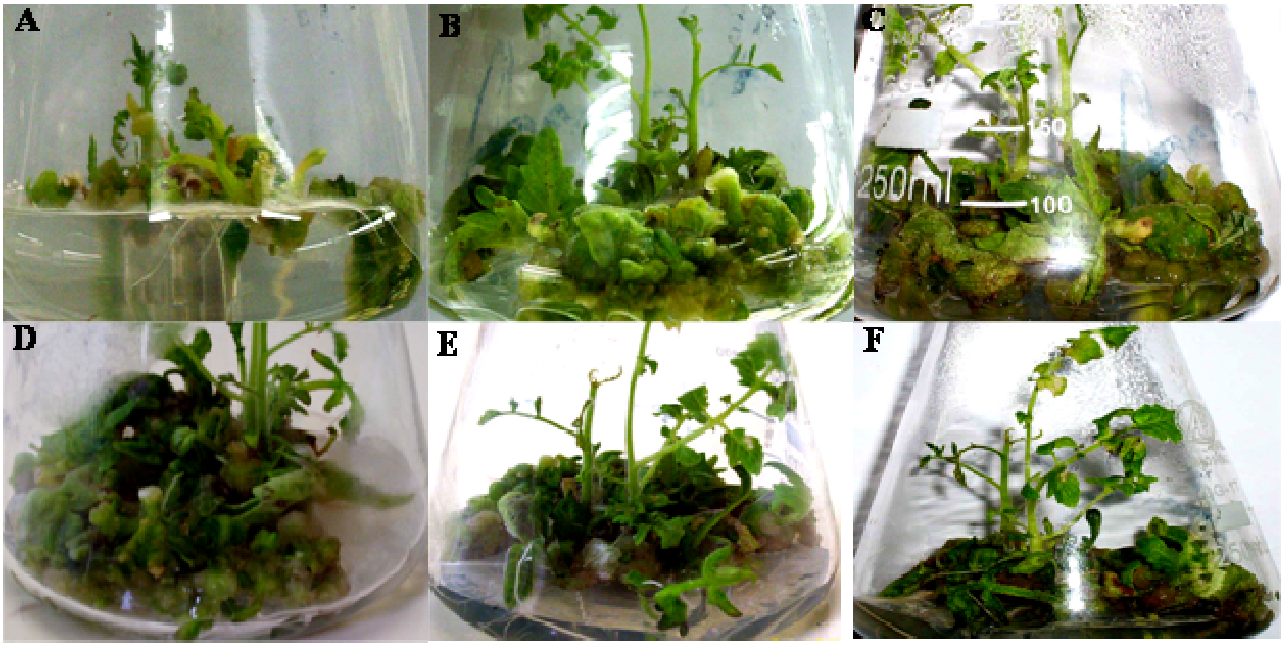

Figure 2. Callus morphogenesis to shoots 15 days after culturing with CW. (A) Avinash hypocotyls derived multiple regeneration, (B) Pant Bahr leaf disc derived regeneration on RM_8, (C and D) Pusa Ruby hypocotyl derived callus on medium RM_8 medium, (E) Pant Bahr morphogenesis on RM_7 medium and (F) Roma leaf disc derived regeneration on RM_8 medium.

Maximum number of shoot primordial was obtained with RM_8 followed by RM_9 treatment in all the varieties for hypocotyls as well as for leaf disc. However, less number of shoot primordial were developed from leaf discs in all the varieties. Pant Bahr produced the minimum number of shoot primordial in RM_2 for hypocotyls and for leaf discs. Significantly, higher number of shoot primordial was observed with the addition of CW in the regenerative media as against the media without CW. On average basis irrespective of the explant source, Avinash produced maximum number of shoot primordial followed by Rio Grande and were statistically similar to each other. Roma ranked 3rd for production of shoot primordial followed by Pant Bahr and Pusa Ruby. Statistically, significant and maximum number of shoot primordial was produced at the RM_8 which was followed by RM_9 and RM_7 (Table 10). Figures 1E, F, H and 2 represented the multiple shoot primordial with CW in tomato varieties tested. After acclimatization the regenerated plants were shifted to the glass house and Table 11 represents the survival percentage of plants which is clearly more from the media containing CW.

Table 8. Analysis of variance for number of shoot primordial obtained in different genotypes of tomato.

K value	Source	D.F	S.S	M.S	F value	Probability
1	Replication	2	0.50	0.25	2.21238	0.11213
2	Genotypes (A)	4	35.358	8.839	78.2814	0.0000
4	Explant (B)	1	673.613	673.613	5961.177	0.0000
6	AB	4	7.537	1.884	16.6871	0.0000
8	Treatment (C)	9	917.470	101.941	902.7885	0.0000
10	AC	36	80.831	2.245	19.8845	0.0000
12	BC	9	46.050	5.117	45.3129	0.0000
14	ABC	36	55.374	1.538	13.6220	0.0000
-15	Error	198	22.358	0.113		
	Total	299	1839.091			

Table 9. Comparison of number of shoot primordia of hypocotyls and leaf disc in tomato varieties on MS regeneration media supplemented with or without CW along with 3% sucrose with IAA 0.5 mg L^{-1}, kinetin, 4 g L^{-1} of gelrite.

Media	Hypocotyls						Leaf discs					
	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean
RM_1	4.20 ^{E}	5.25 ^{D}	4.41 ^{DE}	4.03 ^{EF}	4.30 ^{F}	4.44 ^{G}	2.23 ^{DE}	2.2 ^{C}	1.25 ^{E}	1.20 ^{F}	1.15 ^{G}	1.62 ^{E}
RM_2	6.53 ^{C}	5.30 ^{D}	4.50^{CDE}	3.88 ^{F}	5.76 ^{E}	5.197^{EF}	2.50 ^{CDE}	2 ^{CD}	1.26 ^{E}	1.12 ^{F}	1.70 ^{FG}	1.719 ^{E}
RM_3	7.76 ^{B}	5.26 ^{D}	6.00 ^{B}	6.26 ^{B}	6.75 ^{CD}	6.41 ^{C}	3.00 ^{C}	2 ^{CD}	2.24 ^{D}	2.10^{DE}	2.21 ^{EF}	2.315^{D}
RM_4	5.46 ^{D}	6.10 ^{C}	5.13^{CD}	4.46 ^{EF}	6.50^{CDE}	5.53^{DE}	1.95 ^{E}	1.2 ^{D}	1.18 ^{E}	1.58^{EF}	2.76 ^{DE}	1.750^{E}
RM_5	6.40 ^{C}	4.00 ^{E}	3.76 ^{E}	5.23^{CD}	6.03 ^{DE}	5.08 ^{F}	2.85 ^{CD}	1.5 ^{D}	1.75^{DE}	1.51^{EF}	1.71 ^{FG}	1.865 ^{E}
RM_6	5.13 ^{D}	5.5 ^{CD}	4.18 ^{E}	5.68 ^{BC}	5.75 ^{E}	4.45 ^{G}	2.133 ^{DE}	1.8^{CD}	2.46^{CD}	3.26 ^{C}	3.10 ^{D}	2.56 ^{D}
RM_7	6.76 ^{C}	6.10 ^{C}	4.41 ^{DE}	4.66 ^{DE}	6.76 ^{CD}	5.74^{DG}	3.033 ^{C}	3.6 ^{B}	3.00 ^{C}	3.36 ^{C}	4.467 ^{C}	3.50 ^{C}
RM_8	10.77 ^{A}	11.0 ^{A}	10.6 ^{A}	11.5 ^{A}	11.00 ^{A}	11.0 ^{A}	6.933 ^{A}	8.5 ^{A}	7.433 ^{A}	6.38 ^{A}	7.883 ^{A}	7.427 ^{A}
RM_9	7.88 ^{B}	7.03 ^{B}	5.19 ^{C}	5.63 ^{BC}	8.33 ^{B}	6.81 ^{B}	4.467 ^{B}	4.2 ^{B}	5.10 ^{B}	5.03 ^{B}	7.017 ^{B}	5.17 ^{B}
RM_{10}	5.51 ^{D}	5.73^{CD}	5.20 ^{C}	5.56 ^{BC}	6.97 ^{C}	5.79 ^{D}	2.383 ^{CDE}	2.4 ^{C}	2.33 ^{B}	2.83^{CD}	2.993 ^{D}	2.60 ^{D}
Mean	5.70 ^{C}	6.41^{AB}	6.64 ^{A}	6.13 ^{B}	5.34 ^{C}		2.80 ^{B}	2.83 ^{B}	3.50 ^{A}	3.15^{AB}	2.97 ^{B}	

Means not followed by the same letter within a group are significantly different at LSD = 0.01.

DISCUSSION

Tomato is a major vegetable crop and it is grown in almost every country of the world. Development of protocols for *in vitro* selection can provide new advances for the production of stress tolerant varieties. Although, some information is available on the morphogenesis of tomato, the techniques have not been developed to a level at which they can be utilized in large-scale multi- plication of commercially important varieties. The morphogenesis response seems to be highly dependent plant

Table 10. Number of shoot primordia of five tomato varieties on MS regeneration media supplemented with or without CW along with 3 % sucrose with IAA 0.5 mg L^{-1}, kinetin, 4 g L^{-1} of gelrite.

Treatment	Rio Grande	Roma	Pusa Ruby	Pant Bahr	Avinash	Mean
RM_1	3.217 ^{F}	3.578 ^{D}	2.833 ^{EF}	2.617 ^{E}	2.725 ^{F}	3.03 ^{F}
RM_2	4.517 ^{D}	3.650 ^{D}	2.883 ^{EF}	2.50 ^{E}	3.733 ^{E}	3.458 ^{E}
RM_3	5.400 ^{C}	3.625 ^{D}	4.12 ^{C}	4.183 ^{C}	4.483 ^{D}	4.363 ^{CD}
RM_4	3.708 ^{EF}	3.683 ^{D}	3.158 ^{EF}	3.02 ^{DE}	4.633 ^{D}	3.642 ^{E}
RM_5	4.625 ^{D}	2.750 ^{E}	2.758 ^{F}	3.372 ^{D}	3.875 ^{E}	3.476 ^{E}
RM_6	3.633 ^{EF}	3.708 ^{D}	3.32 ^{DE}	4.467 ^{C}	2.392 ^{EF}	3.505 ^{E}
RM_7	4.900 ^{CD}	4.883 ^{C}	3.708 ^{CD}	4.017 ^{C}	5.617 ^{C}	4.625 ^{C}
RM_8	8.850 ^{A}	9.758 ^{A}	9.05 ^{A}	8.975 ^{A}	9.422 ^{A}	9.215 ^{A}
RM_9	6.175 ^{B}	5.633 ^{B}	5.148 ^{B}	5.333 ^{B}	7.675 ^{B}	5.993 ^{B}
RM_{10}	3.950 ^{E}	4.100 ^{D}	3.767 ^{CD}	4.200 ^{C}	4.980 ^{D}	4.199 ^{D}
Mean	4.898 ^{A}	4.555 ^{B}	4.075 ^{C}	4.269 ^{C}	4.956 ^{A}	

Means not followed by the same letter within a group are significantly different at LSD = 0.01.

Table 11. Survival percentage of plantlets for different tissues in tomato.

Variety	Media	Tissue	% Survival
Rio Grande	–ive C.W	Hypocotyl	42.8
Rio Grande	+ive C.W	Hypocotyl	60
Rio Grande	–ive C.W	Leaf Disc	56.6
Rio Grande	+ive C.W	Leaf Disc	65.8
Roma	–ive C.W	Hypocotyl	45.6
Roma	+ive C.W	Hypocotyl	59.5
Roma	–ive C.W	Leaf Disc	42.8
Roma	+ive C.W	Leaf Disc	50.0
Pusa Ruby	–ive C.W	Hypocotyl	37.4
Pusa Ruby	+ive C.W	Hypocotyl	47.8
Pusa Ruby	–ive C.W	Leaf Disc	31.6
Pusa Ruby	+ive C.W	Leaf Disc	42.5
Pant Bahr	–ive C.W	Hypocotyl	30.0
Pant Bahr	+ive C.W	Hypocotyl	42.5
Pant Bahr	–ive C.W	Leaf Disc	27.0
Pant Bahr	+ive C.W	Leaf Disc	33.33
Avinash	–ive C.W	Hypocotyl	46.6
Avinash	+ive C.W	Hypocotyl	56.11
Avinash	–ive C.W	Leaf Disc	37.4
Avinash	+ive C.W	Leaf Disc	42.5

growth regulators used in the media, which is again cultivar and genotypic specific. To overcome these problems certain modern approaches of gene manipulation might be required, in which *in vitro* regeneration of the transformed cells is an important prerequisite. A good *in vitro* plant regeneration system may also assist in further improvement of the commercially important varieties for disease resistance via genetic engineering. The development of a cost effective and efficient protocol for mass propagation of high quality seedlings via tomato tissue culture could help lower the price per seedling. Despite the potential and vast amount of the research undertaken on this subject, plant tissue culture has not become an integral part of tomato-breeding programmes. As no other viable alternatives were offered so far, zeatin remains in use in recent research work in tomato micropropagation (Gao et al., 2009; Jabeen et al., 2009; Abu-El-Heba et al., 2008; Qiu et al., 2007; Prematilake et al., 2002), despite

its high price, which is an effective limitation to the commercial use of developed protocols. Efforts to replace zeatin on the *in vitro* culture protocols by single synthetic cytokinins, such as BAP or kinetin were not very successful as they did not prove to allow good proliferation rates andusually they induced explant hiperhidricity.

In the present trials, zeatin was successfully replaced by CW and Kinetin. CW is known as a natural substance with high levels of zeatin in its composition and found in the last years an increased importance in micropropagation protocols of economically important species, such as *Arachnis labrosa* (Temjensangba and Deb, 2005), sorghum (Baskaran et al., 2006) and Dendrobium (Puchooa, 2004). Coconut milk was also shown to stimulate cell division in other cultured tissues due to the presence of cytokinins and its use as a supplement was adopted in many laboratories in citrus and Dendrobium (Mukhtar et al., 2005; Roy, 2008). As it happens with other reports on zeatin replacement, where the association of different cytokinins is presented as the best way to improve the multiplication rates, also in this trial the single effect of CW was not sufficient to promote satisfactory multiplication and best results were obtained with a blend of CW and Kinetin. In order to achieve quickest regeneration with maximum number of shoot primordia CW was used in combination with IAA and kinetin. From hypocotyls and leaf disc, maximum regeneration was observed also in RM_8 medium with CW. Maximum regeneration was observed in Avinash 95.65% and 91.43% in Rio Grande in hypocotyls in RM_8 medium with 12% CW. By keeping the IAA concentration (0.5 mg L^{-1}) constant any change in the concentration of kinetin in 1.5 mg L^{-1} decreased the regeneration in both hypocotyls and leaf discs. Significant increase in regeneration percentage was recorded with hypocotyls over leaf discs, as kind of tissue and media combinations or hormonal balance has strong effects on callus induction. Maximum regeneration percentage was recorded in Avinash variety followed by Rio Grande and Roma. Sheeja et al. (2004) found that CW (5%) and use of young hypocotyls explants were able to enhance plantlet regeneration of tomato cultivars, but the method is complex; not direct regeneration but callus induction within 7 - 10 days and after 30 - 40 days regeneration is achieved with lot of adjuvant such as folic acid, biotin, glucose, sucrose, agar, agarose, etc and the glass house data of survival of plants was not described. Whereas in this study we have achieved regeneration quickly within 12 - 15 days with leaf discs and 20 - 25 days with hypocotyls and the survival of the plants obtained by using CW was more rather than the plants produced without it. Lower days to maturity could be very useful for the *in vitro* regeneration and also for the transgenic plantlets because this parameter decreased the soma clonal variations in order to maintain the genetic stability of the genotypes. In all the media combinations tested, number of shoot primordial increased significantly with inclusion of CW in comparison to without CW. This increase in the development of leaf primordial is very important for the *in vitro* regeneration and to obtain more number of transgenic plantlets from the same callus. Bhatia and Ashwath (2008) utilized the adjuvants such as activated charcoal and ascorbic acid to produce longer shoots utilizing tomato cotyledons. Paramesh et al. (2010) cultured cotyledon leaf and hypocotyls of tomato variety L15 with 3 mg L^{-1} kinetin + 0.3 mg L^{-1} IAA for callus induction and 5 mg L^{-1} kinetin for regeneration in transformation experiments, but cultivars tested here did not respond well solely with IAA and kinetin, and CW seems to be very important adjuvant.

Other complex plant juices and liquid endosperms have been shown to possess stimulatory properties more or less similar to those of coconut water. These include liquid endosperm from immature corn, tomato juice immature fruits and seeds, orange juice, malt extract, yeast extract, casein hydrolysate, leaf extracts, sap from a number of plants and tumour extracts (Netien et al., 1951; Straus and La Rue, 1954; Steward and Shantz, 1959). In all the future transformation experiments, RM_8 will be applied because of maximum, quickest regeneration with multiple shoot primordial for transformation experiments.

Conclusions

A full protocol for micro propagation of the tomato varieties was presented. The data revealed multiplication compatible for the mass propagation of the cultivar, with one *in vitro* rooted plant being produced in 15 - 25 days with multiple shoot primordial. Using significantly lower-price compounds to replace zeatin on all the micro propagation stages, the major objectives of the trials were achieved. The only concern with the use of CW in commercial micro propagation protocols is the stability of the zeatin concentration in its composition. During the 2-year experimental period filter-sterilized freshly available coconut water was used, different lots were tested and the results always presented a very high regularity. Nevertheless, to significantly reduce media fees, coconut water directly taken from fresh coconuts was used. The protocol here presented is now being tested on other economically important tomato cultivars.

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