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In Vitro Regeneration of Tomato (Lycopersicon esculentum Mill.) Role of Metatopolin, Benzyladenine and Casein hydrolysate

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Abstract
The effect of adding metatopolin (mT), benzyladenine (BA) individually to the nutrient medium at equal molar concentration (1 mM) and their interaction with three concentrations (0.1, 0.3 & 0.5 $g.l^{-1)}$ of casein hydrolysate (CH) on *in vitro* regeneration of Tomato (*Lycopersicon esculentum* Mill.) cv. Super marmande were studied. mT caused a significant increase in leaves number and adventitious shoots number compared with BA, while BA exceeded mT significantly in shoots length.The results show that there were no significant differences between mT and BA regarding roots number and root length. Both BA and mT caused induction of callus at the base of microshoots. CH at 0.1 $g.l^{-1}$ and its interaction with mT caused a significant increase in leaves number and adventitious shoots number whereas its interaction with BA caused a significant increase in shoot length. Increasing CH concentration and its interaction with either mT or BA caused a gradual significant decrease in callus fresh weight when compared with the control.
Keywords: Metatopolin, Benzyladenine, Casein hydrolysate, Tomato, Adventitious shoots, Roots, Callus.

1. Introduction

Tomato (*lycopersicon esculentum* Mill.) is an important Solanaceous vegetable crop grown throughout the world for its versatile uses. It is one of the most important protective foods as it possesses appreciable quantities of vitamins and minerals and sometime rightly referred to as poor man's orange (Devi *et al.* 2008). In Iraq tomato is important vegetable crop cultivated all over the country throughout the year.

Since the discovery of topolins as natural occurring aromatic cytokinins (CKs), they have emerged as genuine alternatives to the long serving CKs such as benzyladenine, zeatin and kinetin in plant tissue culture (Aremu *et al.* 2012). Topolins, especially the metatopolin (mT) and its derivatives have been employed for culture initiation, protocol optimization and for counteracting various *in vitro* induced physiological disorders in many species. During micropropagation of many plant species however, physiological disorders such as stunted growth, epigenetic and somaclonal variation are often encountered (Bairu *et al.* 2011; Smulders and de Klerk 2011). These problems reduce the commercial application of plant tissue culture (PTC) protocols in several plant species. Moreover, the choice of CK remain critical to the success or failure of any micropropagation endeavour (Werbrouck, 2010; Amoo *et al*. 2011). Researchers are continuously searching for new as well as superior CKs. Since the discovery of mT and its derivatives as naturally occurring aromatic CKs in several plant species (Jones *et al.* 1996; Tarkowska *et al.* 2003), shoot multiplication rate, alleviating physiological disorders, better acclimatization and rooting are making topolins popular amongst plant tissue culturists.

Although 6-benzyladenine (BA) is the most commonly used cytokinin in tissue culture protocols, an increasing number of publications have shown the effectiveness of topolins in optimising protocols for increased shoot production and controlling tissue culture- induced problems that are common with the use of BA (Werbrouck *et al*. 1995, 1996).

Application of additives like activated charcoal (AC), casein hydrolysate (CH), coconut milk (CM) & silver nitrate (AgNO3) & their impact is adapted to the cultural needs (Vinod *et al.* 2009) i.e. objectives of the experimental studies like micropropagation, regeneration, cytodifferentiation, androgenesis, biosynthesis of secondary metabolites and biotransformation of cells as well as the particular plant species taken.

Casein hydrolysate (CH) is an organic nitrogen supplement containing a mixture of amino acids. Being a good source of reduced nitrogen it has been widely used as an additive to embryo culture media (Narayanswamy, 2007). It has proved superior to the combined effect of the amino acids. CH can be a source of calcium, phosphate, several microelements, vitamins and, most importantly, a mixture of up to 18 amino acids (George and de Klerk, 2008). Several investigators have concluded that CH itself is more effective for plant culture than the addition of the major amino acids which it provides. In prepared mixtures of amino acids resembling those in CH, competitive inhibition between some of the constituents is often observed (Monlar *et al.* 2011).

The author detected a lake in studies about the effect of mT and its interaction with CH on tomato in Iraq and this study may be the first one which deal with this subject. So the present study aims to investigate the role of mT and BA individually at equal molar concentration and their interaction with CH on different stages of *in vitro* regeneration of tomato (*Lycopersicon esculentum* Mill.) cv. Super marmande.

2. Materials and methods

2.1 Chemicals

Plant growth regulators (PGRs) 6-benzyladenine (BA), metatopolin [6-(3-hydroxybenzylamino) purine (mT)], MS powder (Murashige and Skoog,1962), Gamborg vitamins (Gamborg *et al*.1968), Casein hydrolysate (CH), Agar, Tween 20 were obtained from Phytotechnology Laboratories, USA.

2.2 Plant material

Seeds of tomato (*Lycopersicon esculentum* Mill.) cv. Super marmande were obtained from the Directory of Agriculture, Basrah province. The seeds were rinsed thoroughly in running tap water, and surface sterilized in 70% alcohol (v/v) for 1 min followed by 2% sodium hypochlorite (v/v) with Tween 20 for 20 min. then rinsed in sterile distilled water 3 times before transferring to Pyrex test tubes (150x20 mm) containing 10 ml MS basal medium alone solidified with 6 gm.l^{-1} agar. The shoot tips were excised from one week old seedling (Fig. 1A) under sterile conditions in the growth room's laminar air flow cabinet and used as explants in the subsequent experiments.

2.3 Treatments and growth conditions

The shoot tips (Fig.1B) were inoculated on media consisting of MS basal salts supplemented with Gamborg vitamins (1 mg.l^{-1}), sucrose (30 g.l^{-1}), BA or mT at equal molar concentration (1mM) and CH (0.1, 0.3, 0.5 g.l^{-1}). The control treatment was the medium contain either BA or mT free of CH.

The medium was adjusted to pH 5.8 with 0.1 M of KOH or HCl before adding the gelling agent (6 g.l^{-1} agar) and autoclaving at 121°C and 0.1 MPa for 20 min. Large diameter culture tubes (50X100 mm) contained 25 ml of MS medium (Fig.1C) and 10 replicates per treatment were used. Subculture was made after 4 weeks and the microshoots produced were transferred to glass jars (65x130 mm) with plastic cover (Fig.1D) and the callus (which formed at the base of the microshoots) were excised and weighed. The experiment continued till the formation of plantlets each of which was transferred to Pyrex test tubes (150x20 mm, Fig1E) contain 10 ml of the media with the same previously described constituents except that the amount of agar was minimizes to 3 g.l^{-1} in order to fix the roots smoothly. The experiment continued for 12 weeks after which the data were recorded.

2.4 Experiment parameters

The following parameters were estimated as average for each treatment and calculated at the end of the experiment (after 12 weeks) except callus fresh weight which calculated after 4 weeks.

-Callus fresh weight (g) by mean of electronic Sartorius BL2105 balance.
-Mean Plantlets leaves number.
-Mean plantlets adventitious shoots number.
-Mean plantlets shoot length (cm).
-Mean plantlets adventitious roots number.
-Mean plantlets root length (cm).

2.5 Statistical analysis

The experiments were designed as a completely randomized factorial with 2 factors (type of PGR X CH concentrations) with ten replicates for each treatment. Data were subjected to a two way analysis of variance (ANOVA) using Genstat programme, means were compared using Revised Least Significant Difference (RLSD) at $P<0.05$.

3. Results

3.1 Effect of mT and BA and their interaction with CH on:

3.1.1 Mean number of leaves:

Table 1 shows the effect of mT, BA and their interaction with CH on mean number of leaves. The highest number of leaves (29.5) were observed in the interaction between mT and 0.1 g.l^{-1} CH which exceeded significantly all the other treatments. A significant increase in leaves number was recorded at 0.1 gm.l^{-1} CH (26.45) as mean for PGRs treatment while 0.5 g.l^{-1} CH caused a significant decrease in leaves number when compared with control. mT caused a significant increase in leaves number when compared with BA (24.25 & 21.15 respectively).

Table1: effect of mT, BA and CH on mean number of leaves in Tomato cv. Super marmande

PGR(1mM)	CH ($g.l^{-1}$)				
	0.0	0.1	0.3	0.5	mean
mT	24.2 ± 2.9	29.5 ± 3.7	23.1 ± 3.3	21.3 ± 1.9	24.52
BA	20.0 ± 2.2	23.4 ± 2.7	22.0 ± 2.1	19.2 ± 2.0	21.15
mean	22.10	26.45	22.55	20.25	
RLSD: interaction= 4.80, PGR= 1.20, CH= 2.41					

3.1.2 Mean shoots number and mean shoot length:
Results in table 2 indicated that mT caused a significant increase in mean adventitious shoots number (6.02) when compared with BA (4.55). CH at 0.1 $g.l^{-1}$ caused a significant increase in mean shoot number compared with control. The highest number of shoots was recorded at the interaction between 0.1 $g.l^{-1}$ CH and mT.

Table2: effect of mT, BA and CH on mean adventitious shoots number of Tomato cv. Super marmande

PGR(1mM)	CH ($g.l^{-1}$)				
	0.0	0.1	0.3	0.5	mean
mT	6.1 ± 0.85	7.5 ± 0.95	5.2 ± 0.80	5.3 ± 0.85	6.02
BA	4.9 ± 0.52	5.6 ± 0.55	4.3 ± 0.33	3.4 ± 0.52	4.55
mean	5.5	6.55	4.75	4.35	
RLSD: interaction=1.1, PGR= 0.27, CH= 0.55					

Table3: effect of mT, BA and CH on mean shoot length (cm) of Tomato cv. Super marmande

PGR(1mM)	CH ($g.l^{-1}$)				
	0.0	0.1	0.3	0.5	mean
mT	8.9 ± 0.53	10.1 ± 0.59	8.3 ± 0.60	7.4 ± 0.61	8.67
BA	9.4 ± 0.35	10.6 ± 0.32	8.8 ± 0.42	7.2 ± 0.34	9.00
mean	9.15	10.34	8.55	7.3	
RLSD: interaction=N.S, PGR= N.S, CH= N.S					

Regarding mean shoot length results in table 3 indicated that BA exceeded mT significantly in plantlets mean shoot length.

The highest shoot length was recorded at interaction between BA and 0.1 $g.l^{-1}$ CH (10.6 cm). CH at high concentrations (0.3 & 0.5 $g.l^{-1}$) caused a significant decrease in shoot length compared with control.

3.1.3 Mean number of roots and mean root length:
Table 4 shows that the differences between mT and BA was insignificant regarding the mean roots number. The addition of CH to the nutrient medium and the interaction between PGRs and CH was also insignificant in concern to mean number of roots.

Table 4: effect of mT, BA and CH on mean roots number of Tomato cv. Super marmande

PGR(1mM)	CH ($g.l^{-1}$)				
	0.0	0.1	0.3	0.5	mean
mT	26.9 ± 2.80	26.3 ± 0.63	26.4 ± 0.86	26.2 ± 0.56	26.45
BA	27.6 ± 2.44	28.1 ± 0.23	27.6 ± 0.48	27.3 ± 0.15	27.65
mean	27.25	27.2	27.0	26.79	
RLSD: interaction=N.S, PGR= N.S, CH= N.S					

Table 5: effect of mT, BA and CH on mean root length (cm) of Tomato cv. Super marmande

PGR(1mM)	CH ($g.l^{-1}$)				
	0.0	0.1	0.3	0.5	mean
mT	4.3 ± 0.41	5.2 ± 0.72	4.8 ± 0.33	4.4 ± 0.51	4.67
BA	3.9 ± 0.39	4.7 ± 0.22	4.6 ± 4.1	4.5 ± 0.44	4.42
mean	4.10	4.95	4.70	4.45	
RLSD: 0.70, PGR= 0.17, CH= 0.35					

Results in Table 5 indicated that CH at 0.1 $g.l^{-1}$ caused a significant increase in root length and a significant decrease at 0.5 $g.l^{-1}$ when compared with control. The table showed also that the differences between mT and BA and the interaction between PGRs and CH were insignificant regarding root length.

3.1.4 Callus fresh weight (g)

Results in table 4 revealed that there was no significant difference between BA and mT in the mean fresh weight of the induced callus. Addition of CH caused a gradual significant decrease in callus fresh weight especially at 0.5 $g.l^{-1}$ when compared with the control.

Table 6: effect of mT, BA and CH on callus fresh weight (g) of Tomato cv. Super marmande

PGR(1mM)	CH ($g.l^{-1}$)				
	0.0	0.1	0.3	0.5	mean
mT	0.41 ± 0.14	0.33 ± 0.14	0.26 ± 0.13	0.15 ± 0.09	0.28
BA	0.49 ± 0.25	0.36 ± 0.21	0.31 ± 0.11	0.17 ± 0.06	0.33
mean	0.45	0.34	0.28	0.16	
RLSD: interaction=0.51, PGR=0.09, CH=0.19					

4. Discussion

In the present study larger leaves number (table 1), adventitious shoots number (table 2) were produced with mT compared to BA at equimolar concentrations suggesting that the levels of irreversibly sequestrated cytokinins were lower in the mT treated plants (Moyo *et al.*2011).

Shoot regeneration and multiplication during micropropagation is affected by the type and concentration of applied PGRs, especially the CKs due to their importance in cell division and organogenesis (Howell *et al.* 2003). BA remains one of the most effective CK that promotes *in vitro* shoot regeneration and multiplication of plant species. In an attempt to address various physiological and developmental problems associated with CKs in general and BA in particular however, other CKs are being tested (Arema *et al.*2012).

Various studies on a wide range of plant species highlighted the potential of topolins as a substitute to the commonly used CKs. Using equimolar concentration (10.0 µM) of either mT or BA, Werbrouck *et al.* (1996) observed better shoot–root balance with mT- treated *Spathiphyllum floribundum* plantlets. Similarly, mT at various concentrations produced more shoots compared to either BA or Zeatin during the micropropagation of *Aloe polyphylla* (Bairu *et al.* 2007). In banana cv. 'Williams', the use of topolins (mT, mTR, MemT, MemTR) at 7.5, 15 and 30 µM had higher shoot multiplication rates than BA (Bairu *et al.* 2008).

The results in table 4 and 5 revealed that the differences between mT and BA in concern to number of roots and root length was insignificant. The induction of adventitious roots is a critical and complex process in

micropropagation that is primarily influenced by the type and concentration of auxin used (Hatzilazarou *et al.* 2006). The formation of adventitious roots under the influence of auxins is associated with the process of dedifferentiation, in which cells switch to a new morphogenetic pathway and produce root primordia (Hatzilazarou *et al.* 2006). While auxins are known to promote rooting, the role of cytokinins in rhizogenesis remains controversial, though they are generally regarded as rooting inhibitors (Feito *et al.* 1996).

Several studies on different species have reported that the type and concentration of CKs have a profound effect on in vitro plant acclimatization competence (Moncaleán *et al.* 2001; Bairu *et al.* 2008; Valero-Aracama *et al.* 2010). In addition, some authors have reported that CKs generally have inhibitory effects on rooting, resulting in poor acclimatization rates afterwards (Werbrouck *et al.* 1995; Bairu *et al.* 2008). The interaction of CKs with auxins has always been an important consideration in PTC.

Regarding callus fresh weight the results in table 6 indicated that there was no significant difference between BA and mT in the induction of callus. However, a significant reduction in callus response was observed with casein hydrolysate. The same result was obtained in tomato by Bhatia and Ashwath (2008).

CH especially at concentration of 0.1 g.l and it interaction with mT have a positive effect on leaves number, shoot number and shoot length. CH is an organic nitrogen supplement containing a mixture of amino acids it has been widely used as an additive to cultures (Bansal and Gokhale, 2012). It has proved superior to the combined effect of amino acids mixture (Narayanswamy, 2007). It has been thought that nitrogen deficiency can cheaply fulfilled by its addition (Al-Khayri, 2011).

Presumably it contain some stimulatory factors yet undefined. CH caused a reduction in callus fresh weight and has no significant effect on roots number and root length, this may resulted from the lake of exogenously addition of auxins which are effective in rhizogenesis and calugenesis. The synergistic effect of PGR's and additives has been demonstrated in several medicinal plants a report on stevia (Sridhar, and Aswath, 2014) regeneration fortification of CH resulted with good regeneration responses and multiplication of shoots was increased two fold. Similar research findings were reported on *Oroxyllum indicum* (Bansal. and Gokhale, 2012) regeneration using CH as growth additive. CH enhances the direct shoot regeneration and the number of shoots found to multiply 9.34 fold over control treatments at 20 mg/L CH is well documented. CH has also been found useful in *Anogeissus pendula* and *A. latifolia* (Saxena, and Dhawan, 2001) *in vitro* studies. The synergistic effect of PGR's and additives has been demonstrated in several medicinal plants.

5. Conclusion

Fortification of culture media with different plant growth regulators i.e. auxins and cytokinins is not enough to regenerate the plant with high efficiency. This type of cultures in some cases may be improved by using the proper type and concentration of PGRs, incorporation of additives in the media due to their growth and development promoting activities. In the present work additive used was CH which lead at concentration of 0.1 $g.l^{-1}$ and with interaction with 1mM of mT to enhance leaves number, adventitious shoots regeneration and reduced in the quantity of callus induced at the base of plantlets leading to further growth inhibition. So we found that using mT as a cytokinin has a superiority on BA and the interaction between 1mM mT with CH at 0.1 $g.l^{-1}$ is of great importance in the micropropagation of tomato with high efficiency through organogenesis.

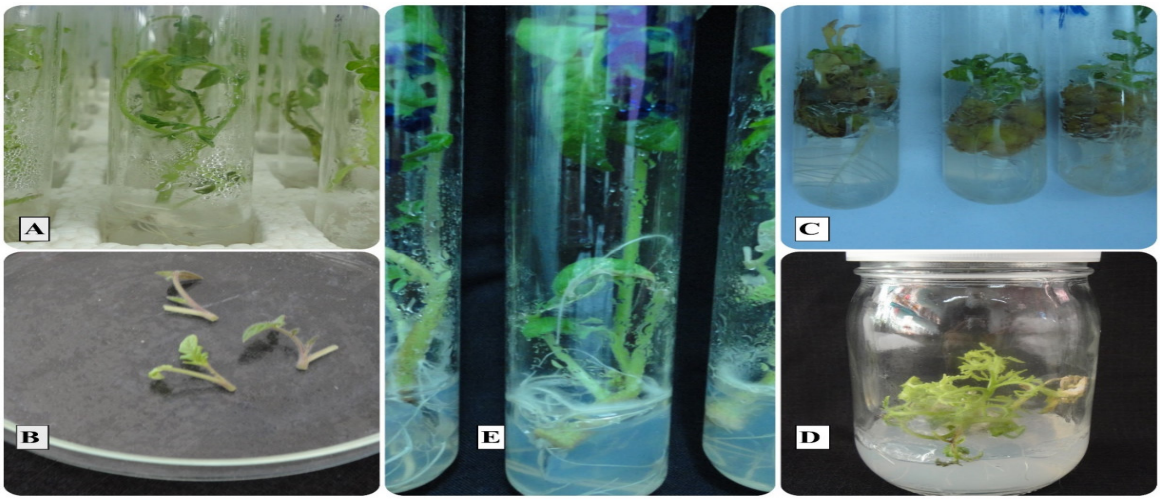

Figure1. 1-Microprpagation stages of Tomato cv. Supermarmand. A-seedlings 7 days old. B-Excied shoots tips. C- Callus at the bottom of micro shoots. D- Adventitious shoots with leaves and roots. E- Plantlets at the end of experiment.

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