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Establishment of Plant Regeneration Protocol in Cucumber: Fruit Juices Used as Carbon Source

Ikram-ul Haq[1], Zahra Arshad[2], Taseer[3]

Institute of Biotechnology and Genetic Engineering (IBGE),, University of Sindh, Jamshoro-76080, Pakistan

Abstract: ***The effect of commercial fruit juices was assessed on callus induction, its proliferation and plant regeneration in cucumber (Cucumissativus*L.*) cv., Liza. Almost 3-4 mm, cuttings of seedling (7-10 days old) were cultured for callus induction on MS_2 [MS_0 (Murashige& Skoog, 1962) basal salts, 0.6 mg L^{-1} 2,4-D (2,4-Dichlorophenoxy acetic acid), 0.4 mg L^{-1} BAP (Benzyleaminopurine)] medium and also on simple MS_0 medium supplemented with fruit juices in place of 3% sucrose like as MS_{2a} (MS_0 with orange juice), MS_{2b} (apple), MS_{2c} (red grapes), MS_{2d} (strawberry) for callus induction. No callusing was observed on fruits juice cultures while callus proliferation was higher in orange juice culture (MS_{2a}), when 3-weeks old callus sub-cultured from MS_2 medium. Maximum plant regeneration (3.98±0.65 per callus) was observed on $^{*}MS_{2c}$ medium when somatic embryogenesis was induced on MS_3 (0.1 mg L^{-1} BAP, 0.5 mg L^{-1} glutamate) medium in dark (2-weeks). Root induction efficiency was higher in $^{1}MS_{2b}$ and $^{1}MS_{2d}{}^{1}/_{2}MS$) cultures under light condition. After plant hardening, they were transferred to soil conditions.***

Keywords: Cucumissativus L., callus induction, fruit juices, somatic embryogenesis, plant regeneration.

1. Introduction

Cucumber (*Cucumissativus*L.) is a most important vegetable vine crop (Kadans, 1979). Unfortunately, this crop has a narrow genetic base, which hampers the transfer of new desired traits from closely related species through crossing (Den *et al*., 1990). Aseptic genetic transformation for improvement of cucumber can be applied. This gene technology is dependent on its reproducible tissue culture system for the regeneration of complete numerous plantlets from primary explants (Gamborg*et al*., 1968). However, no universal and applicable cheapest method of plant regeneration is available for all species because tissues from one genotype will behave differentially under *in-vitro*.

Aseptic plant regeneration from a single cell of the callus cultures remains very important for crop improvement. Meanwhile, somatic embryogenesis has been considered as a most important mode of indirect regeneration of plantlets (Williams and Maheswaran, 1986; Kuehnle*et al*., 1992; Chen and Kuehnle, 1996; Beyramizade*et al*., 2008; Yu et al., 2009). In spite of plant micropropagation, encapsulated somatic embryos may also be stored easily and transferable to long distances. Meanwhile plant regeneration via somatic embryogenesis is only a way for plant improvement through genetic engineering as well as for prevention of chimerasim (Ghosh and Sen, 1994; Ozgen*et al*., 1998; Delporte*et al*., 2001; Zale *et al*., 2004; Yu *et al*., 2008; Shah *et al*., 2003).

Carbohydrates are substrate of respiration and precursors of many compounds as well as being the building blocks of various macromolecules in the plant cells. Similarly, it may be involved in several biological processes among the cells (Gibson and Bruck, 2000; Smeekens, 2000). Most common source of carbohydrate for aseptic plant cultures is sucrose and resident in phloem tissues dominantly (Zimmermann and Ziegler, 1975; Gray *et al*., 1993; Lou *et al*., 1995). Both sources of carbohydrates and its concentrations have been affecting the differentiation processes during vegetative growth or even initiation of growth (somatic embryos) of the developing plantlets from cells or tissues under aseptic conditions (Kamada*et al*., 1989; Lou *et al*., 1997; Nakagawa *et al*., 2001; Arslan*et al*., 2002; Jabeen*et al*., 2005). Fruits are rich sources of sugars and vitamins in nature. These components can also be useful for plant tissue growth as a source of carbohydrates as well as osmotic agents in the nutrient medium. Abundance of carbohydrates in juice extracts of fruits might be useful in culture medium to acts as growth enhancers by triggering the differentiation in cultured tissues into somatic embryos (Kinnersley and Henderson, 1988; Siddique and Paswan, 1998; Waseem*et al*., 2008).

The aim to conduct, this present study was to establish an indirect plant regeneration protocol for cucumber (*Cucumis-sativus*L.) cv., Liza. Growth promoting activity of various fruit juices was assessed on indirect plant regeneration in cucumber by using in place of sucrose in culture medium. This established protocol can be useful for other cucumber cultivars with certain modification in concentration and types of juices (orange and red-grapes) in near future.

2. Materials and Methods

Healthy seeds of cucumber (*Cucumissativus*L.) cv., Liza were selected and surface sterilized with 70% ethanol for 1 min. They were stirred for 20 min in 30% commercial bleach (5% NaOCl) than followed by three time rinses with sterile distilled water. They were placed on Whatman-4 filter paper to dry surplus water but kept moisturized until they were cultured on plant nutrient medium. The sterilized seeds were cultured on MS_0 [Murashige and Skoog (1962) basal nutrient salts supplemented with B_5 vitamins (Gamborg*et al*., 1968) and 3% sucrose] medium for seed germination. Culture was incubated at 25°C±1, first in dark for 2-days and then in light conditions.

Young stem-cuttings (3-4 mm) of almost 7-10 days old seedlings were used as explants for callus induction. They were cultured on **MS_2** (MS_0, 0.6 mg L^{-1} 2, 4-D; 0.4 mg L^{-1} BAP) and various fruit containing medium in place of sucrose in MS media like as **MS_{2a}** (MS_0, 158 ml L^{-1} orange

juice), **MS_{2b}** (MS_0, 130 ml L^{-1} apple juice), MS_{2c} (MS_0, 100 ml L^{-1} red grapes juice), **MS_{2d}** (MS_0, 195 ml L^{-1} strawberry juice) for 3-weeks (Table 1 & 2). These added amounts of each juice were used in place of sucrose as it contains exactly 30 g sucrose.

Three weeks old callus (~100mg) from MS_2 medium was sub-cultured on all of the media used for callus induction, as described above. After 3-weeks, rate of callus proliferation was determined by applying formula as given below;

$$\text{Callus proliferation (\%):} \quad \frac{Callus\ Final\ Wt - Callus\ initial\ Wt}{Callus\ Final\ Wt} x100$$

For somatic embryogenesis, almost 6-weeks old, well proliferating calluses from MS_2 culture were sub-cultured on MS_3 (MS_0, 1.0 mg L^{-1} BAP; and 0.5 mg L^{-1} glutamate) liquid medium under dark conditions for 10-days.

After somatic embryogenesis, calluses were cultured on $^*MS_2$ (MS_0, 1.0 mg L^{-1} BAP) as well as on fruit juice containing media ($^*MS_{2a}$, $^*MS_{2b}$ and $^*MS_{2c}$), here * is symbol of medium used at plant regeneration stage. Plant regeneration cultures were incubated under light conditions for 3-weeks.

Regenerated plantlets were sub-cultured on $^1MS_2$ ($^1/_2MS_0$, 0.05 mg L^{-1} IBA) and other juice containing media ($^1MS_{2a}$, $^1MS_{2b}$, $^1MS_{2c}$ and $^1MS_{2d}$) for root induction (here ^{1} is a symbol for plant rooting stage) from $^*MS_2$ culture (Table 1 & 2). Some of growth parameters of regenerated plantlets like as number of plantlets per callus, root induction rate, plant height and root length were measured. Rooted plantlets were washed with tap water and subjected for plant hardening and then transferred to soil conditions.

The pH of each medium (except somatic embryogenesis) was adjusted between 5.7-5.8 and solidified with 1% agar agar. These media were autoclaved at 121ºC and 15 Lbs $inch^{-2}$ pressure for 15 minutes. The cultures maintained at 25^{O}C ± 1, with $\frac{16}{8} hrs$ photoperiod (25 mmol m^{-2} s^{-1}). For dark conditions temperature remained same but light period off.

Almost 5-7 explants and 100 mg callus per treatment were maintained. Data were presented as mean with its standard error. The ANOVA was computed for data significance and treatments were tested by applying Duncan's multiple range tests at 5% level of difference.

Table 1: Chemical composition of different fruit juices used for plant regeneration through somatic embryogenesis in cucumber (*Cucumissativus*L.) cv., Liza

#s	Medium	Juice composition (1.0 g D wt)
a.	**Orange** ***MS_{2a}***	0.3% vit A, 1.56% vit C, 1.5% riboflavin, 3.4% niacin, 1.9% Ca, 1.3% Fe, 3.4% vit B6, 1.5% folate, 3.2% P, 5.3% Mg, 3.1% pentathenate
b.	**Apple** ***MS_{2b}***	12% sugars, 7% fructose, 2.7% sucrose, 2% glucose, 0.7% malic acid, 0.5% pectin, 0.5% starch, 0.01% polyphenols, 0.006 proteins, 0.0005% vitamins (mainly ascorbic acid), 0.02% ashes, 0.003% β-carotene.
c.	**Red-Grapes** ***MS_{2c}***	25% carbohydrates, 0.17% nitrate, 0.01% protein, 0.11% free amino acids, 0.002% humin, 0.6% Al, 0.007% B, 0.025% Ca, 0.01% Cl, 0.0003% Cu, 0.003% Fe, 0.025% Mg, 0.005% Mn, 0.25% K, 0.05% P, 0.001% Rb, 0.0002% Si, 0.02% Na, 0.003% SO_4.
d.	**Strawberry** ***MS_{2d}***	0.4% protein, 0.15% fat, 12.5% carbohydrate, 0.2% fiber, 10.42% sugar, 1.1% Ca, 0.026% Fe, 1 Mg, 1.2% P, 0.4% Na, 10% K, 0.007% Zn, 0.005% Cu, 2.5% vit C, 0.006% thiamine, 0.003% riboflavin, 0.028% niacin, 0.006% vit B6, 8.78 vit A, 0.004% vit E, 0.05% vit K.

3. Results

The 3-4 mm micro-stem cuttings of 7-10 days old seedling of cucumber () cv., Liza were cultured for callus induction. During three weeks of callusing culture, callus induction was observed on MS_2 medium only, not others as supplied with different fruit juices in place of sucrose in MS_2callusing cultures i.e. MS_{2a}, $MS_{2b,}$ and MS_{2c}. Almost 3-weeks old callus from MS_2 medium sub-cultured on those same media, which used for callus induction. After 3-weeks of culture, maximum callus proliferation was observed in cultures supplied with orange juices (MS_{2a}) as compared to other juices containing medium as well as including hormone based MS_2 culture also (Table 2).

Figure 1: Establishment of indirect plant regeneration in cucumber (*Cucumissativus*L.) cv., Liza. a: Almost 7-10-

days old seedling on MS_0 medium; **b:** 3-weeks old callus developed in micro-stem cuttings of seedlings on MS_2 medium; **c**: Well proliferating callus culture on MS_{2a} (orange juice supplied) after 6 weeks; **d:** Callus proliferation (turning to greenish) after somatic embryo induction (MS_3) under light conditions; **e:** Development of plantlets on $^{*}MS_{2c}$ nutrient culture with grapes juices after 2-week under light conditions; **f**: 3-4-weeks old rooted plantlet on $^{1}MS_{2d}$ (strawberry juice) medium.

For somatic embryogenesis, 6-weeks old calluses that were proliferated on MS_2 medium was sub-cultured on MS_3 medium, while others were refreshed on their respective media. These cultures were incubated in dark conditions for 10-days. All calluses produced somatic embryos which were cultured on MS_3 (supplied with BAP and glutamate) medium. In media supplied with fruit juices (without hormones) remained without induction or development of somatic embryos and become succulent non-embryogenic calluses.

Table 2: Effect of different fruit juices on indirect plant regeneration in cucumber (*Cucumissativus*L.) cv., Liza

Medium	Callus proliferation (%)	# of plantlets per callus	Rooting (%)	Plant height (cm)	Root length (cm)
$^{*,1}MS_2$	^{b}44.65±2.42	3.42±0.33	^{b}65.45±2.80	^{a}3.54±0.45	^{c}4.62±0.65
$^{*,1}MS_{2a}$	^{a}62.23±1.42	2.41±0.88	^{e}21.46±3.01	^{a}3.68±0.64	^{b}5.24±0.05
$^{*,1}MS_{2b}$	^{d}18.32±1.25	-	^{d}24.51±3.45	^{c}2.42±0.34	^{e}3.87±0.18
$^{*,1}MS_{2c}$	^{c}38.97±1.22	3.98±0.65	^{c}45.51±2.65	^{b}2.88±0.51	^{a}6.42±0.42
$^{*,1}MS_{2d}$	^{d}14.42±1.05	-	^{a}87.54±2.54	^{c}2.34±0.42	^{d}4.11±0.64
DMR	4.81***	-	3.09***	0.25***	0.27***

MS_2: Used for callusing; $^{*}MS_2$: Used for plant regeneration; $^{1}MS_2$ (1/2 MS_0): Root induction; ***: Significance $p>0.05$; ^{a}, ^{b}, ^{c}, ^{d}: Juices of orange, apple, red-grapes and strawberry respectively in MS_0 medium (Table 1).

When all of these differentiated callus cultures (with somatic embryos) from MS_3 medium were sub-cultured on $^{*}MS_2$ (MS_3 without glutamate) medium and incubated under light conditions. Plant regeneration was observed on $^{*}MS_2$ medium as well as on orange and red grape juices supplied cultures within 3-weeks. When the somatic embryos were induced on MS_3 medium and then cultured on fruit juice containing medium. Higher plant regeneration efficiency was observed in $^{*}MS_{2c}$ cultures (Table 2). Plant regeneration was also observed among the cultures supplied with other juices also ($^{*}MS_{2a}$ and $^{*}MS_{2b}$).

The regenerated plantlets were cultured on $^{1}MS_2$, $^{1}MS_{2a}$, $^{1}MS_{2b}$ and $^{1}MS_{2c}$ ($^{1}MS_2$: 1/2MS_0 with sucrose, $^{1}MS_{2a}$, $^{1}MS_{2b}$, &$^{1}MS_2$: 1/2MS_0 with juices) media. Among these cultures efficient root induction was observed on $^{1}MS_{2b}$ and $^{1}MS_{2d}$, while almost absent in $^{1}MS_{2a}$ and $^{1}MS_{2c}$ cultures. While maximum plant height was observed in $^{*}MS_{2a}$ cultures and root length in $^{1}MS_2$ and $^{1}MS_{2d}$ cultures.

4. Discussion

A large number of factors have been documented that could be associated with callus induction and its proliferation like as auxins (Merkle*et al*., 1995), while aseptic growth is mainly dependent on concentration of various growth regulators in the medium. In this study, cultures supplemented with juices are unable to induce callus in the cultured explants. However, higher callus multiplication rate observed on MS_{2a}, when 3-weeks old calluses sub-cultured from MS_2 medium (Fig 1). Among the multiplying callus, no cell differentiation occurs, either cultured on fruit juice supplied (i.e. MS_{2a}, MS_{2b} and MS_{2c}) or hormone added (MS_2) medium.

Somatic embryogenesis in cucumber is achievable when 2,4-D is replaced with glutamate and concentration of BAP reduced to 0.1 mg L^{-1} in callus development medium (MS_2). After somatic embryogenesis, plant regeneration is possible. Regenerated plantlets had been observed on the hormones supplied cultures as well as among the juice supplied cultures also (Table 2). Grapes juice has considered as best component for shoot development in the embryogenic calluses. Regeneration efficiency remain significant in $^{*}MS_{2c}$ medium than others supplied with hormones or nutrient cultures with fruit juices ($^{*}MS_{2a}$; $^{*}MS_{2b}$).

From this study, it should be concluded that callus induction and somatic embryogenesis in calluses are largely controlled by the presence of a specific typed hormones with specific concentrations in plant nutrient cultures. All juices have performed significantly role as an incremental growth precursors but not involved to trigger any turning mode of growth. Fruit juices are best for callus proliferation and plant regeneration from somatic embryos. They also involved in root induction in regenerated plantlets. In this protocol, by using fruit juices plant regeneration efficiency increased significantly. By using this valuable protocol, Liza cultivar of cucumber as well as other related cultivars can be improved against various targeted environmental stresses and also for other potential food crops.

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