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Full Length Research Paper

Influence of media and growth regulators on regeneration and morphological characteristics of strawberry cvs Kurdistan and Merck (*Fragaria* x *ananassa* Duch.)

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To evaluate the best media and growth regulator combinations for the regeneration and morphological characteristics of strawberry, meristems of Kurdistan and Merck strawberry cultivars were cultured on Murashige and Skoog (1962), Gamborg et al. (1968) and Nitsch and Nitsch (1969) media supplemented with various growth regulator combinations including B1: (BA 1.0 mg/l + IBA 0.05 mg/l + GA3 0.05 mg/l), B2: (Kinetin 5 mg/l + 2,4-D 0.5 mg/l + GA3 0.05 mg/l) and B3: (BA 2 mg/l + IBA 0.2 mg/l + GA3 0.05 mg/l). The largest number of shoots was obtained on NN medium supplemented with B1 followed by MS and B5 media with the same growth regulator combination. In the media, B2 increased shoot elongation rate in both cultivars, but caused a low frequency of shoot formation. There was significant difference among various media and growth regulator combinations in regards to morphological traits such as leaf length, width of terminal leaflets and number of terminal leaflets teeth, whereas little differences were observed in the length of terminal leaflets and the length of terminal leaflet petiolate.

Key words: Strawberry, growth regulator, regeneration, cultivar.

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most important fruit plants for both fresh consumption and food processing in the temperate and subtropical areas, with a global production of over 4.1 million tons and a production area of about 255000 ha (FAO, 2008). It is traditionally propagated vegetatively by rooted runners (Biswas et al., 2008). This method was not suitable due to the incidence of many diseases infection. Moreover, the conventional method of production is not adequate to meet the commercial demand. Karhu and Hakala (2002) and Singh et al. (2004) observed that micropropagated strawberry plants were comparatively better in different characters (crown size, number of runners, flowering time and yield of berries) than conventionally propagated runner plants.

Strawberries are affected by over 30 viruses and phytoplasmas, many of which can greatly reduce yield, rapidly spread in the field, and may not cause obvious symptoms (Martin and Tzanetakis, 2006). The yield reduction caused by some viruses may be up to 80% (Thompson and Jelkman, 2003). Therefore the runners of strawberry are not always suitable for this type of cultivation due to their vulnerability and susceptibility to pathological agents. The use of meristem culture for virus elimination is employed for a number of species.

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Furthermore, meristem regenerated plants usually maintain the genetic characteristics of the parent plant (Nehra and Kartha, 1994). Meristems, generally obtained from runners of virus-free plants, are commonly used to establish *in vitro* cultures, which are employed for mass propagation or as a source of plant material for regeneration and transformation experiments (Boxus, 1992). Micro-propagation has been extensively used for the rapid production of many plant species and cultivars (Hartmann et al., 2002) and several studies have attested the tissue cultured plants being more advantageous than those by conventional propagation in terms of fruit yield, vigor, the number of runners and leaves per plant (Zebrowska et al., 2003). However, despite its extra-ordinary potential, this technology is still confronted with many problems. The occurrence of variation in plants regenerated from *in vitro* cultures has been reported for morphological and yield variation in micro-propagated strawberries (Graham, 2005).

Although somaclonal variation may be used as a source for variation to get superior clones. In this regard, resistances of *in vitro*-derived plants to fungal pathogens (Sowik et al., 2001; Hammerschlag et al., 2006), low temperature (Rugienius and Stanys, 2001) and salt stress (Dziadczyk et al., 2003) have been studied with promising results, but it could be also a very serious problem in the plant tissue culture industry resulting in the production of undesirable plant off-types (Karp, 1993; Cassells, 1999). These variants pose a problem for production of uniform, true-to-type plants. Nehra et al. (1994) found that two cultivars of strawberry responded differently to various forms of *in vitro* propagation and in both cases variants were found in callus-derived plantlets, but not those derived from meristems or via direct leaf regeneration. So the aim of this study was to test the influence of media, hormone combinations and cultivar on the regeneration and morphological characteristics of two strawberry cultivars.

MATERIALS AND METHODS

Stock plants of two strawberry cultivars (Kurdistan and Merck) growing in greenhouse were used as explants in these experiments. Daughter plants were harvested and meristems were excised from terminal buds on the crowns. Shoot tip of about 1 to 2 cm were excised and washed under running tap water for 45 min to remove surface contaminants. Surface sterilization was done inside the laminar air flow cabinet by 30 s dipping the explants in 70% (v/v) ethanol and for 15 min in aqueous solution of 1% (v/v) sodium hypochlorite. After four washes in sterile double distilled water, each meristem is excised aseptically, in a laminar air flow transfer hood using a scalpel and forceps under stereoscope. Shoot tip of about 2 to 3 mm were again surface sterilized in sodium hypochlorite (0.25%) for 1 min and rinsed 2 - 3 times with sterile water. The wound sites exposed to sterilization agent were trimmed and the meristem, about 0.2- 0.5 mm long, with two leaf primordia were cultured on Murashige and Skoog (MS), Gamborg et al. (1968) (B5) and Nitsch and Nitsch (NN) media supplemented with various growth regulator combinations including B_1: (BA 1.0 mg/l + IBA 0.05 mg/l + GA_3 0.05 mg/l), B_2:(Kinetin 5 mg/l + 2,4-D 0.5 mg/l + GA_3 0.05 mg/l) and B_3:(BA 2 mg/l + IBA 0.2 mg/l + GA_3 0.05 mg/l). All the media were solidified with 8 g/l Agar (Sigma). The pH of medium was adjusted to 5.8 using 0.1 N NaOH or HCl before autoclaving. The media was autoclaved for fifteen minutes at 121°C and 15 psi. All cultures were incubated in a growth chamber under 16/8 h light/dark cycle at 25±1°C under 35 µ mol m^{-2} s^{-1} illumination provided by cool-white fluorescent lamps.

Twenty meristems were used for each combination. The experiment was repeated twice. The results of the experiment were analyzed statistically using a standard statistical procedure with factorial design using the SAS® statistical analysis software with proc GLM and means separation via Duncan's multiple range tests.

RESULTS AND DISCUSSION

To evaluate the best media and growth regulator combinations for the regeneration and morphological characteristics of two strawberry cultivars were tested on many types of growth media with a strictly defined mineral composition, e.g. basal salt mixtures according to Murashige and Skoog (1962), Gamborg et al. (1968), Nitsch and Nitsch (1969) and growth regulator combinations B_1: (BA 1.0 mg/l + IBA 0.05 mg/l + GA_3 0.05 mg/l), B_2:(Kinetin 5 mg/l + 2,4-D 0.5 mg/l + GA_3 0.05 mg/l) and B_3:(BA 2 mg/l + IBA 0.2 mg/l + GA_3 0.05 mg/l). (Table 1).

It is evident (Table 1) that there is a strong and intricate interaction between plant growth regulators, culture conditions and cultivar. Several parameters used during the *in vitro* phase can affect the behavior of micro-propagated strawberry, e.g. plant genotype, mineral formulation, type and concentration of hormone in the medium (Faedi et al., 2002; Mozafari and Govaorova, 2005). Analysis of variance revealed that the media had highly significant difference ($P<0.01$) in most of the traits. MS medium had high influence on most of traits studied than NN and B5 medium. MS medium is commonly used in the *in vitro* multiplication of strawberry.

Analysis of the study results showed a significant influence of the medium type on the number of shoots formed from an explant. The largest number of shoots was obtained on NN medium followed by MS medium, whereas the lowest number on B5 medium (Table 1). These results confirm the finding of kozak et al. (2007) and Faedi et al. (2002), whose indicated that the chemical composition of the medium is one of the factors determining the success of *in vitro* cultures.

Among the various plant growth regulators supplements used, the best response towards number of shoot regeneration was observed from meristem explants on B_1: (BA 1.0 mg/l + IBA 0.05 mg/l + GA_3 0.05 mg/l). This combination showed the best performance of shoot proliferation in both cultivars. The lowest number recorded on B_2: (Kinetin 5 mg/l + 2, 4-D 0.5 mg/l + GA_3 0.05 mg/l) (Figure 1). Selection of the proper hormone

Table 1. Effect of various media and growth regulator combinations on different traits in the meristem explant of two strawberry cultivars (Merck and Kurdistan).

Media	Growth regulator	Traits and cultivars					
		Number of shoots per explant		Shoots height (cm)		Leaf length (cm)	
		Kurdistan	Merck	Kurdistan	Merck	Kurdistan	Merck
MS	B_1	1.86^{d}	0.42^{d}	4.46^{cd}	4.66^{cd}	5.63^{c}	5.10^{cd}
	B_2	1.66^{e}	2.46^{b}	5.33^{a}	6.70^{a}	4.63^{d}	7.30^{a}
	B_3	2.50^{c}	2.44^{b}	4.73^{b}	5.00^{bc}	7.30^{a}	5.50^{c}
B5	B_1	0.78^{g}	2.16^{c}	4.23^{d}	4.30^{c}	6.60^{b}	4.90^{d}
	B_2	1.02^{f}	0.50^{d}	5.16^{ba}	6.50^{a}	4.60^{d}	7.20^{a}
	B_3	1.86^{d}	2.66^{b}	4.66^{bc}	3.78^{e}	5.73^{c}	5.21^{cd}
NN	B_1	3.44^{a}	3.24^{a}	4.26^{d}	6.46^{a}	7.23^{a}	7.13^{a}
	B_2	0.72^{g}	1.96^{c}	5.16^{ba}	6.60^{a}	3.40^{e}	6.36^{b}
	B_3	3.20^{b}	3.18^{a}	4.73^{b}	5.20^{b}	5.33^{c}	6.10^{b}

Means with the same small letter in each column do not significantly differ by Duncan's multiple range tests ($p < 0.05$). B_1: (BA 1.0 mg/l + IBA 0.05 mg/l + GA_3 0.05 mg/l), B_2:(Kinetin 5 mg/l + 2, 4-D 0.5 mg/l + GA_3 0.05 mg/l) and B_3:(BA 2 mg/l + IBA 0.2 mg/l + GA_3 0.05 mg/l).

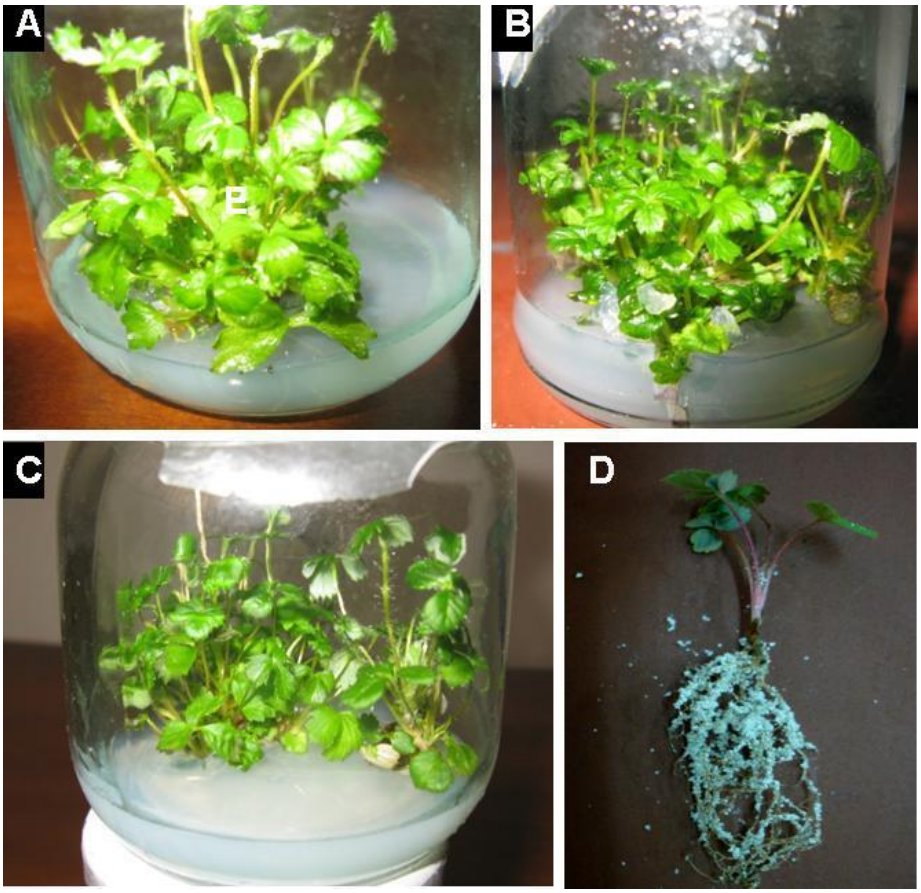

Figure 1. Effect of various growth regulator combinations on different traits in the meristem explant of strawberry (A) B_1: (BA 1.0 mg/l + IBA 0.05 mg/l + GA_3 0.05 mg/l), (B) B_3:(BA 2 mg/l + IBA 0.2 mg/l + GA_3 0.05 mg/l), (C) B_2:(Kinetin 5 mg/l + 2, 4-D 0.5 mg/l + GA_3 0.05 mg/l) and (D) Rooted plant.

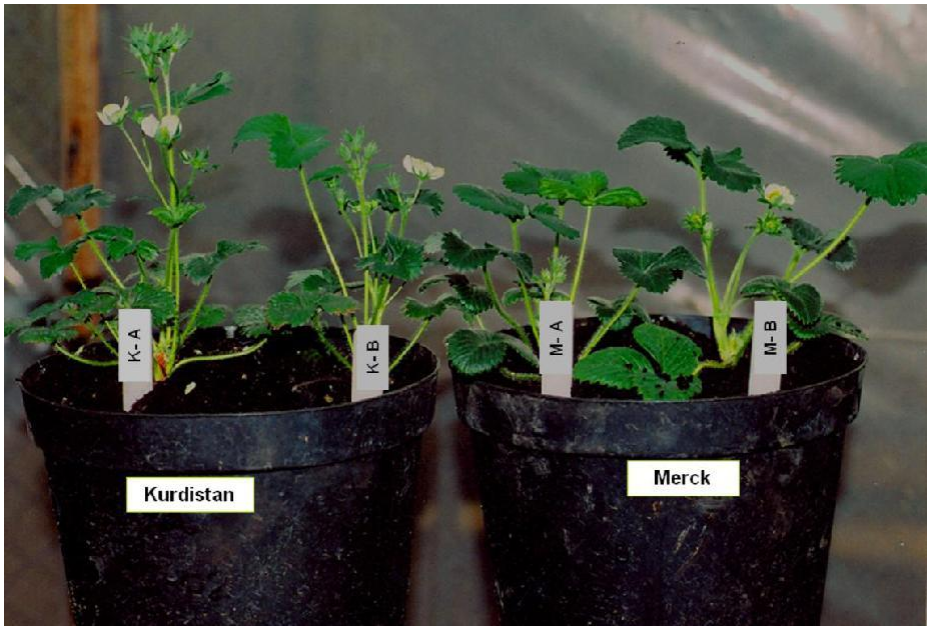

Figure 2. Effect of various media and growth regulator combinations on the phenotypic polymorphism of two strawberry cultivars (Merck and Kurdistan).

combination is the key to successful regeneration of strawberry (Jimenez-Bermudez, 2002; Morozova, 2003; Litwińczuk, 2004).

Two cultivars, Kurdistan and Merck were chosen to compare the effect on shoot multiplication. The results showed shoot multiplication occurred in two cultivars (Table 1). The highest number of shoots per culture (3.44) was also recorded from Kurdistan cultivar. Passey et al. (2003), observed similar effects on strawberry. They testing the regeneration ability of seven commercial cultivars using a range of explants showed that leaf disks gave the highest regeneration rates, although two genotypes showed a limited ability to regenerate shoots in all explants tested. The variation in the regeneration capacity amongst different cultivars has also been observed in other studies (Nehra et al., 1990a; Singh et al., 2004; Gerdakaneh et al., 2009 and 2011), indicating that a strong genetic component determines the success of adventitious regeneration.

Table 1 shows the effect of different concentrations of growth regulator on shoots length in the meristem explant of strawberry cultivars. All growth regulator combinations stimulated shoot formation and stem enlargement for each explant in these treatments. B_2: (Kinetin 5 mg/l + 2, 4-D 0.5 mg/l + GA_3 0.05 mg/l) increased shoot elongation rate in both cultivars. Kinetin in B_2 growth regulator combinations increased shoot elongation rate but caused a low frequency of shoot formation (Figure 1). Kinetin has positive influence in number of shoot formation. Negative correlations were observed between shoots length and number of shoots per explant. Shoot height was negatively correlated with number of shoots (Table 1). Some workers reported high concentration of BAP is the best for strawberry micro propagation (Morozova, 2003) while other authors suggested 1.0 mg/l IAA + 1.0 mg/l BAP + 0.05 mg/l GA_3; 0.5 mg/l BA + 0.1 mg/l GA_3 + 0.1 mg/l IBA (Boxus, 1999; Litwińczuk, 2004) and 0.5 mg/l BA + 0.1 mg/l IBA (Bozena, 2001) for strawberry micro propagation.

Two meristem culture-derived strawberry cultivars showed variations at morphological level. The phenotypic polymorphism was clearly observed in the leaf and inflorescence morphology of the regenerated strawberry plants (Figure 2). Tables 1and 2 shows significant difference among various media and hormone combinations in regards to morphological traits such as leaf length, width of terminal leaflets and number of terminal leaflets teeth. Commercial production of strawberry using micro propagation processes bears several risks. Plant off-types, that is, non true-to-type and genetically not identical to the mother plant, may be among the resulting plants (Kunert et al., 2003). The extent of variation depends on genotype, age of the donor plant, explant type and plant hormones in the culture medium (Arnholdt-Schmitt et al., 1995; Gupta, 1997; Jain, 1997).

There were little differences in terminal leaflet shape, length of the terminal leaflet petiolate, teeth shape of the terminal leaflet and the terminal leaflets length. However, through micro-propagation of strawberries, several morphological abnormalities were detected as

Table 2. Effect of various media and growth regulator combinations on leaf morphological traits in the meristem explant of two strawberry cultivars.

Media and growth regulator		Traits and cultivars							
		LTL(mm)		WTL(mm)		LTLP(mm)		NTLT	
		Kurdistan	Merck	Kurdistan	Merck	Kurdistan	Merck	Kurdistan	Merck
MS	B_1	9.13^{c}	12.06^{b}	7.33^{cd}	12.33^{b}	0.93^{bc}	1.13^{b}	4.90^{f}	7.56^{b}
	B_2	8.60^{c}	8.03^{f}	6.93^{d}	7.06^{f}	1.00^{ab}	0.73^{d}	8.76^{a}	6.03^{de}
	B_3	9.13^{c}	7.96^{f}	8.58^{b}	6.16^{g}	0.93^{bc}	1.10^{bc}	6.63^{e}	6.16^{d}
B5	B_1	8.30^{cd}	8.53^{ef}	7.66^{c}	5.96^{g}	1.04^{a}	0.10^{e}	7.30^{d}	5.56^{e}
	B_2	10.33^{b}	15.66^{a}	7.00^{d}	10.56^{c}	0.13^{e}	1.26^{a}	7.33^{d}	7.76^{b}
	B_3	11.40^{a}	9.33^{cd}	8.26^{b}	9.23^{d}	0.10^{e}	1.13^{b}	8.23^{bc}	6.23^{d}
NN	B_1	10.53^{ab}	9.10^{ed}	8.20^{b}	8.13^{e}	0.90^{c}	1.06^{bc}	8.53^{ab}	8.60^{a}
	B_2	7.50^{d}	15.23^{a}	6.33^{e}	13.56^{a}	0.13^{e}	1.05^{c}	5.16^{f}	6.86^{c}
	B_3	11.30^{ab}	9.90^{c}	9.30^{a}	9.03^{d}	0.26^{d}	1.06^{bc}	8.00^{cd}	8.33^{a}

Means with the same small letter in each column do not significantly differ by Duncan's multiple range tests ($p < 0.05$). LTL = Length of terminal leaflets, WTL= Width of terminal leaflets, LTLP= Length of terminal leaflet petiolate, NTLT = Number of terminal leaflets teeth.

somaclonal variation. There are concerns about genetic changes resulting from strawberry micro-propagation. Genetic stability during micro-propagation is controlled by numerous factors including genotype, presence of chimeral tissue, explant type and origin, media type, types and concentrations of growth regulators, culture conditions (temperature, light, etc.) and duration of culture (Graham, 2005).

In conclusion, we demonstrated that media and growth regulator combinations can affect on the regeneration and morphological characteristics of strawberry. Variation is a very serious problem in the plant tissue culture that depends on cultivar, explant type, media, type and concentration of hormone. Our results showed a much higher level of genetic variability among some morphological traits. While low variation was observed among the other morphological traits, which indicated some morphological traits have high genetic stability.

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