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Effect of Antimitotic Agent Colchicine on *In Vitro* Regeneration of Watermelon

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Abstract

***In vitro* cultures of watermelon were treated with antimitotic agent colchicine to induce ploidy alterations, particularly the induction of tetraploids. Explants cotyledon, embryonic end of seed, transverse sections of epicotyl and hypocotyl were cultured on MS media supplemented with BA (1 μM) and colchicine (0.01%, 0.05% and 0.1%). Explants were subcultured on colchicine free media after 4 and 7 days. Colchicine had negative effect on *in vitro* regeneration but this exhibited explants related response. However, hypocotyl section of seedlings induced maximum callus on 0.01% colchicine. Shoot proliferation was more in cotyledon explants cultured on colchicine (0.01%) for four days. Maximum root induction and root number were recorded in embryonic end explants. Overall, cotyledon and embryonic end explants, and low colchicine concentration (0.01%) was found optimal in watermelon regeneration.**

Key words: *Citrullus lanatus*, BA, callus, polyploid, tetraploid

Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai.] is an important Cucurbitaceous vegetable. In Pakistan it is grown over an area of 20,000 hectares with a production of 420,000 MT (FAO 2004). Seedlessness is an important and desirable breeding goal in horticultural crops. In watermelon polyploidy is utilized to produce seedless fruits. Seedless watermelons are triploids and result from crossing a tetraploid seed parent with a diploid pollen parent (Kihara 1951; Andrus et al. 1971). There is much interest in seedless watermelons in the consumer market (Marr and Gast 1991). Polyploid watermelons are also found to be resistant to watermelon fruit blotch (*Acidivorax avenae* subsp. *citrulli*) and nematodes (Garret et al. 1995). Moreover, in triploid watermelons orange flesh turns into deeper orange color as it ripens and flavor can even improve after harvesting. Also, their tough sunburn-resistant rind makes them excellent for long-distance shipping.

Difficulties with seed germination have retarded the expansion of triploid cultivation. In other cases, the lack of disease resistance was regarded as a serious obstacle. High seed cost has generally attributed to difficulties in obtaining a sufficient number of tetraploid plants as they exhibit low fertility and generally require at least 8-10 years of self pollination before enough plants are obtained for commercial triploid seed production (Compton and Gray 1992). Moreover, *in vivo* treatment of colchicine results in a mixed population of diploid, tetraploid, aneuploids and scrotal and periclinal chimeras (Koh 2002). The induction of tetraploid *in vitro* offers an alternative method to obtain tetraploid plants because of reduction in number of aberrant plants produced and also reduce the time span required for triploid seed production (Compton and Gray 1992; Compton et al. 1993). The objective of the study was to regenerate tetraploid watermelon *in vitro* for breeding seedless watermelons because of their potential in local market and export as triploid seed or seedless watermelon to bring in foreign exchange.

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Materials and Methods

Seeds of diploid watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] cv. 'Sugar Baby' were soaked two hours for ease in removing seed coat. The seeds after removing seed coat were surface disinfected for 20 minutes in 1.25% NaOCl plus one drop of Tween 20 followed by three times rinses with sterile double distilled water and were cultured on MS medium (Murashige and Skoog, 1962) supplemented with BA (0, 1 or 5 μM) to test the effect on regeneration. In these studies MS with 1 μM BA showed promising results and the same was further used in medium with colchicine. These seeds were sterilized as previous and cultured on MS media under dark for 5 days to germinate. Transversely cut epicotyl (E_1) and hypocotyl (E_2) sections (1 cm long) of in vitro grown seedlings, cotyledons (E_3) and seed embryonic end (E_4) was used as an explant and cultured on following media formulations for 4 days (D_1) and 7 days (D_2).

M_0 = MS + 1 μM BA (control)
M_1 = MS + 1 μM BA (0.01% colchicine)
M_2 = MS + 1 μM BA (0.05% colchicine)
M_3 = MS + 1 μM BA (0.1% colchicine)

After 4 and 7 days culturing on colchicine added media, the explants were subcultured on MS + 1 μM BA. The cultures were incubated at 25±2℃ under 16 h photoperiod. Each experiment was laid out in complete randomized design with factorial arrangement and data was analyzed according to Steel and Torrie (1980). Data was summarized as percent of explants induced callus, percent of explants produced shoots, number of shoots per explant, percent of shoots produced roots and number of roots per explant. The rooted plantlets were transplanted in pots having leaf manure and sand, and acclimatized under high humidity.

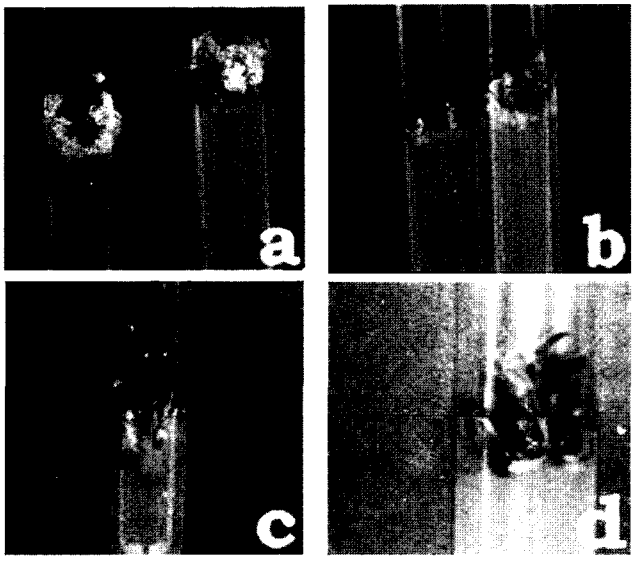

Figure 1. Effect of colchicine media on watermelon regeneration a) callus induction on colchicine (0.01%) from cotyledon and hypocotyl explants b) callus growth of cotyledon explant on 0.1% and 0.01% colchicine media c) adventitious shoot formation on 0.01% colchicine d) shoots regenerated from embryonic end explant.

Results

Effect on callus induction

The comparison of colchicine treatment duration revealed that 4 days treatment yielded statistically more callus induction (54.8%). Transversely cut hypocotyl (E_2) explants produced maximum (90.5% and 90.2%) callus on medium devoid of colchicine (M_0) cultured for 4 days and 7 days, respectively and were statistically non-significant with each other (Table 1). The lowest callus was observed in explants E_1 (14.5%) and E_4 (12.7%) cultured for 7 days on the media containing 0.1% colchicine (Figure 1a,b). Colchicine treatments indicated that callus induction decreased significantly with increased

Table 1. Effect of concentration and treatment duration of colchicine on callus induction in watermelon (all data are in percentages)

Colchicine media (M)	D_1[a]					D_2				
	E_1	E_2	E_3	E_4	Mean(D_1xM)	E_1	E_2	E_3	E_4	Mean(D_2xM)
M_0 0%	40.2	90.5	84.7	34.5	62.5	45.5	90.2	75.5	29.0	60.1
M_1 (0.01%)	34.7	85.2	80.7	30.5	57.8	29.7	75.5	55.5	20.5	45.3
M_2 (0.05%)	30.5	79.5	77.5	28.5	54.0	24.5	69.5	49.2	19.7	40.7
M_3 (0.1%)	20.2	79.2	60.5	19.7	44.9	14.5	65.2	40.7	12.7	33.3
Mean (DxE)	31.4	83.6	75.8	28.3		28.5	75.1	55.2	20.5	
	Mean (D_1) 54.8					Mean (D_2) 44.8				

a: E_1 (transversely cut epicotyl); E_2 (transversely cut hypocotyls); E_3 (cotyledons); E_4 (embryonic end of seeds); D_1 (4 days culture on colchicine); D_2 (7 days culture on colchicine)

concentration in media. Among colchicine treatments, medium with 0.01% colchicine yielded the highest callus (57.8%) followed by medium with 0.05% colchicine on which explants were cultured for four days (54.0%). Both the medium with 0.1% and 0.01% colchicine having explants cultured for 4 and 7 days statistically occupied the same position with 44.9% and 45.3% callus, respectively. The medium containing 0.05% colchicine and the medium with 0.1% colchicine on which explants were cultured for 7 days fall in descending order and showed 40.7% and 33.3% explants response to induce callus, respectively.

The interaction of incubation duration and explant type depicted that transversely cut hypocotyls yielded the highest (83.6%) callus cultured for 4 days on colchicine containing media (Table 1). The same explant cultured for 7 days and cotyledons cultured for 4 days on colchicine added media held statistically second position (75.1% and 75.8%, respectively).

Effect on shoot induction

The percent of explants produced shoots was significantly maximum (17.5%) on media containing 0.01 % colchicine (4 days incubation) when compared treatment duration and media interaction (Table 2). These results show that the highest percentage of explants produced shoots were on control for both treatment durations. The lowest (0.01%) concentration of colchicine, tested for 4 days, regenerated more shoots (Figure 1c). This indicates that both the concentration and incubation duration are equally important for shoot regeneration as the lower concentration of colchicine for longer incubation (7 days) showed same results (10.2%) as 0.05% colchicine on 4 days incubation (10.2%). The least percent of explants produced shoots (3.6%) were at the highest level (0.1%) of colchicine solution treated for 7 days but the same concentration showed statistically higher percentage (7.5%) on 4 days incubation.

The combined effect of treatment duration and explant type presented in the Table 2 indicates that the percent of explants produced shoots were higher (38.9%) for explants E_3 (cotyledons) cultured on colchicine media for 4 days followed by the same explant cultured for 7 days (27.5 %). The explants E_4 (embryonic end) cultured for 4 days induced 16.5% shoots. The other two types of explants induced no shoots on any colchicine treatment. The short incubation (4 days) on colchicine media showed better response of explants to induce shoots (13.9%) than 7 days incubation (9.4%).

The interaction of the three factors studied i.e., explant, media and duration of colchicine treatment is also shown in the Table 2. The highest (55.5% and 54.7%) percent of shoots were recorded in explant E_3 (cotyledons) cultured on colchicine lacking media (M_0). The same explant cultured on M_1 (0.01%) media for 4 days incubation showed 50.2% explants with shoots but higher incubation duration significantly decreased the percent shoots (30.2%). The explant E_4 (embryonic end) showed lesser response than E_3.

Effect on number of shoots

Both the incubation durations of explants on colchicine media were statistically different and produced 3.5 and 3.2 shoots per explant, respectively (Table 3). The colchicine concentration in the medium showed negative effect on number of shoots/explant. The medium with 0.01% colchicine produced 3.9 shoots at D_1 (4 days) but significantly differed with D_2 (7 days) with 3.6 shoots per explant. The lowest number of shoots (2.0) was recorded on M_3 media with 7 days incubation.

The overall interaction of the three factors is presented in the Table 3. It is evident that colchicine lacking media produced more shoots than colchicine added media. The explants E_4 cultured on the lowest colchicine level yielded higher number of shoots (8.5 and 8, respectively) on 4 and

Table 2. Effect of concentration and treatment duration of colchicine on shoot induction in watermelon (all data are in percentages)

Colchicine media (M)	D_1[a]					D_2				
	E_1	E_2	E_3	E_4	Mean(D_1xM)	E_1	E_2	E_3	E_4	Mean(D_2xM)
M_0 (0%)	0	0	55.5	25.2	20.2	0	0	54.7	20.2	18.7
M_1 (0.01%)	0	0	50.2	20.0	17.5	0	0	30.2	10.5	10.2
M_2 (0.05%)	0	0	29.7	11.0	10.2	0	0	14.7	5.0	4.9
M_3 (0.1%)	0	0	20.2	10.0	7.5	0	0	10.5	4.2	3.6
Mean (DxE)	0	0	38.9	16.5		0	0	27.5	10.0	
	Mean (D_1) 13.9					Mean (D_2) 9.4				

a: E_1 (transversely cut epicotyl); E_2 (transversely cut hypocotyls); E_3 (cotyledons); E_4 (embryonic end of seeds); D_1 (4 days culture on colchicine); D_2 (7 days culture on colchicine)

Table 3. Effect of concentration and treatment duration of colchicine on shoot number of explants in watermelon

Colchicine media (M)	D_1[a]					D_2				
	E_1	E_2	E_3	E_4	Mean(D_1xM)	E_1	E_2	E_3	E_4	Mean(D_2xM)
M_0 (0%)	0	0	8.5	9.6	4.5	0	0	8.5	9.6	4.5
M_1 (0.01%)	0	0	7.2	8.5	3.9	0	0	6.5	8.0	3.6
M_2 (0.05%)	0	0	5.4	6.4	2.9	0	0	5.1	6.0	2.7
M_3 (0.1%)	0	0	5.1	5.1	2.5	0	0	4.5	3.5	2.0
Mean (DxE)	0	0	6.5	7.4		0	0	6.2	6.8	
	Mean (D_1) 3.5					Mean (D_2) 3.2				

a: E_1 (transversely cut epicotyl); E_2 (transversely cut hypocotyls); E_3 (cotyledons); E_4 (embryonic end of seeds); D_1 (4 days culture on colchicine); D_2 (7 days culture on colchicine)

Table 4. Effect of concentration and treatment duration of colchicine on root induction from shoots in watermelon (all data are in percentages)

Colchicine media (M)	D_1[a]					D_2				
	E_1	E_2	E_3	E_4	Mean(D_1xM)	E_1	E_2	E_3	E_4	Mean(D_2xM)
M_0 (0%)	0.5	24.7	14.7	100	35.0	0.5	24.7	19.7	100	36.2
M_1 (0.01%)	0.2	15.2	11.5	100	31.5	0.2	11.7	11.0	100	30.4
M_2 (0.05%)	0.0	9.7	10.5	100	30.3	0.0	10.5	7.0	100	29.7
M_3 (0.1%)	0.0	4.7	5.0	100	27.4	0.0	5.7	5.0	100	27.7
Mean (DxE)	0.2	13.6	10.4	100		0.2	13.2	10.7	100	
	Mean (D_1) 31.1					Mean (D_2) 31.0				

a: E_1 (transversely cut epicotyl); E_2 (transversely cut hypocotyls); E_3 (cotyledons); E_4 (embryonic end of seeds); D_1 (4 days culture on colchicine); D_2 (7 days culture on colchicine)

7 days incubation periods (Figure 1d). These two explants on higher levels of colchicine decreased shoots induction capability. The explants E_1 (transversely cut epicotyl) and E_2 (transversely cut hypocotyl) produced no shoots on any media and duration of treatment.

Effect on root induction

Comparison of means of colchicine treatments and culture duration for roots induction (Table 4) represents that the percent of shoots induced root was the highest (36.2%) on media lacking colchicine (M_0) cultured for 7 days. Colchicine (0.01%) media showed significantly higher percent of shoots to induce root (31.5%). Higher concentrations of colchicine in media suppressed the rooting. However, both colchicine treatment durations were statistically similar for root induction.

The explant E_4 (embryonic end) showed similar response to rooting on all the colchicine levels in the medium for both treatment durations i.e., 100% explants gave rise roots and thus formed statistically a one large group (Table 4). The explants E_1 (transversely cut epicotyl) and E_2 (transversely cut hypocotyl) that failed earlier to induce shoot but showed rhizogenesis. Explant E_2 produced more roots than E_3 on both treatment durations. The explants E_1 (transversely cut epicotyl) had 0-0.5% roots on different concentrations of colchicine and duration of colchicine treatment.

Effect on number of roots

The interaction of treatment duration and media type represented highly significant results (Table 5). The higher (2.5 and 2.4) number of roots was formed on control medium on both treatment durations, respectively. The media with 0.01% colchicine yielded maximum roots per explant whereas higher colchicine levels significantly decreased the number of roots. The response of explants exhibited that E_4 (embryonic end) produced the highest (3.6) number of roots per explant on 7 days treatment duration and was statistically alike with 4 days incubation on colchicine media. The explant E_2 (transversely cut hypocotyl) was the next coming explant and produced 2.0 and 2.1 number of roots cultured for 4 and 7 days on colchicine containing media, respectively. Statistically these were similar with explant E_3 (cotyledons) that had 2.2 roots per explant when cultured for 4 days on colchicine added media. Low number of roots per

Table 5. Effect of concentration and treatment duration of colchicine on root number of explants in watermelon

Colchicine media (M)	D_1[a]					D_2				
	E_1	E_2	E_3	E_4	Mean(D_1xM)	E_1	E_2	E_3	E_4	Mean(D_2xM)
M_0 (0%)	1.0	2.0	2.3	4.4	2.4	1.0	2.6	2.0	4.6	2.5
M_1 (0.01%)	0.5	2.0	2.9	4.1	2.2	0.5	2.0	1.1	3.5	1.7
M_2 (0.05%)	0.0	2.0	2.1	3.5	1.9	0.0	1.9	1.0	3.3	1.5
M_3 (0.1%)	0.0	1.9	2.1	2.0	1.5	0.0	1.8	1.0	3.1	1.5
Mean (DxE)	0.4	2.0	2.2	3.5		0.4	2.1	1.3	3.6	
	Mean (D_1) 2.0					Mean (D_2) 1.8				

a: E_1 (transversely cut epicotyl); E_2 (transversely cut hypocotyls); E_3 (cotyledons); E_4 (embryonic end of seeds); D_1 (4 days culture on colchicine); D_2 (7 days culture on colchicine)

explant (0.4) was induced by explant E_1 (transversely cut epicotyl) on both treatment durations.

The interaction of the three factors studied (Table 5) indicates that the embryonic end (E_4) explant cultured for 4 days on 0.01% colchicine media yielded 4.1 number of roots and differed significantly with higher colchicine levels. Minimum number (0-1) of roots was produced by the explant E_1.

Discussion

Use of triploid hybrids has provided a method for production of seedless fruit. Kihara began working on seedless watermelon in 1939 and tetraploid parents have been obtained by treating young diploid seedlings with colchicine (Kihara et al. 1951; Andrus et al. 1971). This treatment produces a limited number of tetraploids but mostly chimeric seedlings. Moreover, the development of triploid cultivars adds several problems to the process of watermelon breeding (Lower and Johnson 1969). Reduced seed vigor and poor germination are the concerns of growers (Maynard 1989). Clonal propagation of tetraploid genotypes for the use of breeding triploid hybrids represents the greatest potential use of micropropagation for watermelon (Gray and Elmstrom 1991). Transversely cut hypocotyl (E_2) explants cultured for 4 days on lower concentration of colchicine in the media induced maximum callus (Figure 1a). The higher colchicine treatment and incubation duration had negative effect on callusing. Firstly, callus induced on injured end of explants and then emerged shoots directly on same media. None of the transversely cut epicotyl or hypocotyl explants induced shoots. The results of present studies are supported by SuYing et al. (1993) and Gao et al. (1996).

Colchicine devoid media induced more shoots than colchicine media as colchicine in media cause stress and mortality (SuYing et al. 1993). As the colchicine treatment duration increased from 4 days to 7 days the number of shoots on each explant significantly decreased. Further it was noted that the number of shoots was more on lower colchicine concentration (Figure 1c) than higher concentration. It indicates that both high level of colchicine and longer treatment durations were toxic for shoot regeneration. However, if higher levels and longer durations produced greater percent of polyploids then their other harmful effects on regeneration can be ignored.

Regeneration of adventitious shoots has been reported from a wide range of diploid watermelon cultivars (Srivastava et al. 1989; Dong and Jia 1991; Compton and Gray 1993). Cotyledons of *in vitro* germinated seedlings were rated as the best source of explants and had high organogenic competence (Compton and Gray 1993). However, improved organogenic competence of cotyledons was reported when seedlings were germinated in darkness (Compton 1999). Higher level of colchicine and longer treatment duration killed shoots just after their initiation. Similarly, Gao et al. (1996) reported that polyploid shoot initials/buds are killed in colchicine media.

Similarly variable response of explants to induce shoots was recorded. The explants cultured to induce shoots showed the best results by the cotyledons (Figure 1b) whose embryonic ends were cut and cultured separately. Compton (2000) found proximal end of cotyledons generally more regenerative. Either whole cotyledon bases or basal halves have been used as explants (Compton and Gray 1993). Both transversely cut sections of hypocotyl or epicotyl showed very poor organogenesis. Similar observations were noted by Srivastava et al. (1989) who found poor regeneration when cultured 0.5 to 1 cm hypocotyl sections. Embryonic ends of seed were the second best explants to regenerate shoots.

In preliminary experiments 1 or 5 μM benzyl adenine (BA) was tested and regeneration was comparatively better on 1 μM BA (data not presented) and the same BA concentration was used with colchicine in media. Media formulation played important role in shoot organogenesis

(Srivastava et al. 1989; Dong and Jia 1991) and colchicine, as expected, had negative effect on *in vitro* regeneration. However, this decreased regeneration exhibited a colchicine concentration and explants related response. Either higher colchicine concentration or longer time duration of treatment resulted in slower growth rates and delayed appearance of organogenesis and similarly it was reported by Väinölä (2000). Optimum shoot regeneration has been achieved by adding 4.410 μM BA (Srivastava et al. 1989; Compton 1999). However, Dong and Jia (1991) reported that adding 2.85 μM IAA improved the number of shoots produced per explant for some genotypes. In contrast 1 μM BA worked well in these studies and it may be due to variable genotypic response to cytokinins.

The effect of colchicine concentration and treatment duration on explant types showed that the percent of shoots produced root were affected by media type and treatment duration in case of embryonic end of seed (E_4) while other explants response was non significant. Ganga and Chezhiyan (2002) also reported reduced rhizogenesis on colchicine or oryzalin media. Transversely cut epicotyl and hypocotyl explants, which earlier did not induce shoots, showed rhizogenesis on callus. Only the transversely cut hypocotyls induced roots on higher colchicine concentration. These results are in alliance with SuYing et al. (1993). Roots induced on the same shoot proliferation media but induction rate was very low. Compton and Gray (1993) reported that shoots can be easily rooted in medium with 1 μM IBA and Dong and Jia (1991) induced roots on 0.54 μM NAA.

The present studies conclude that low colchicine level and cotyledon and embryonic end of seed were better for shoot induction. Nevertheless, colchicine suppressed the regeneration but the low regeneration rate may not be a disadvantage, if the treatment induces desired ploidy.

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