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BREEDING POLYPLOID WATERMELON: INDUCTION, IDENTIFICATION AND SEED GERMINATION OF TETRAPLOIDS

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Abstract

Watermelon is an important vegetable crop world-wide and Pakistan produces over 352 thousand tonnes annually. Genetic improvement to produce seedless watermelon offers high quality fruits. This paper describes about induction of tetraploid breeding parents by mutations, identification of solid tetraploids among a mixed population of treated seedlings and problems related with tetraploid seed germination. Diploid watermelon lines were treated with colchicine (0.2, 0.4, 0.6%) for three successive days at emergence of true leaves. The cultivars responded differently for induction of pure tetraploids; however, showed maximum mortality at 0.6% colchicine. Tetraploid seedlings among treated population were verified by chloroplast counts in guard cell pair of stomata, plant and flower morphology, chromosome counts and DNA quantification by flow cytometry. Tetraploids produced less number of seeds/fruit than those of diploids and had fissures on seed coat along the longitudinal axis. Diploid seeds had complete filled cavity with embryo whereas tetraploids developed weak embryos with some empty cavity and hence lower seed germination. Among different seed germination treatments, seed coat removal and seed nicked at radicle end showed high germination rates (84.3% and 77.1%, respectively). Adherence of thick seed coat to cotyledons in tetraploid seeds was reduced by planting seeds in pots with the radicle end up at 90° angle.

Key words: Polyploidy, tetraploid, germination, colchicine, pollen colpi, FCM

INTRODUCTION

The production of seedless watermelons has been known for about 50 years and commercial cultivars have been available for 20 years. The consumers because of high fruit quality and absence of seed prefer seedless watermelon. Seedless watermelons are triploids (3n=33) produced by crossing a tetraploid seed parent with a diploid (2n=22) pollen parent (Andrus *et al.*, 1971; Kihara, 1951). However, the production of seedless watermelon has been hampered by high seed cost and poor seed germination. High seed cost has generally been attributed to difficulties in obtaining a significant number of tetraploid individuals as they exhibit

low fertility and generally require at least 8-10 years of self pollination before enough individuals are obtained for commercial seed production (Compton & Gray, 1992). Moreover, *in vivo* treatment of colchicine results in a mixed population of diploid, tetraploid, aneuploids and sectoral and periclinal chimeras (Compton *et al.*, 1993).

Polyploids can be induced by applying aqueous colchicine solution to the growing apex of diploid seedlings or by soaking diploid seeds in colchicine solution prior to germination. Most, if not all, tetraploids available have been developed by this method. However, the frequency of tetraploids from such treatments is less than 5% in most cases, and many plants are chimeras. Moreover, fertility of tetraploids developed in this manner is considerably lower than the diploid parents (Stoner & Johnson, 1965). Likewise, spontaneous regeneration of watermelon tetraploids in tissue culture is low (Zhang *et al*., 1995). Dinitroaniline herbicides can be used to generate tetraploid plants from diploid plants of many species in tissue culture (Hansen & Andersen, 1996). Confirmation of tetraploidy can be obtained by comparing the size of the pollen grains (about 1.44 X larger than diploid pollen), and the number of colpi (4 versus 3) (Rhodes & Zhang, 1999). Number of chloroplasts per guard cell pair, ovary diameter, petal and anther diameter, and leaf length by width ratio are also good indicators of plant ploidy (Compton *et al*., 1996). Measurement of the nuclear DNA content by flow cytometry has been suggested as an alternative (Dolezel, 1998). Flow cytometry is a rapid and exact method for estimating nuclear DNA content (Galbraith *et al*., 1983). It can be efficiently used for ploidy determination in plants growing in the field and in the greenhouse (Joachimiak *et al*., 2001; Sliwinska & Steen, 1995) and has already been well established in watermelon (Koh, 2002).

Poor seed germination in polyploid watermelon is generally correlated with thick seed coat, poor embryo and high moisture content (Grange *et al.*, 2000). In many seeds, germination can be inhibited by mechanical restriction exerted by the seed coat. Permeability limitation of water and gases is typical of hard seed coats, but is not uncommon in thin seed coat seeds (Hyde, 1954). Oxygen impermeability has been proposed as an explanation for the germination failure of seeds (Come & Tissaoui, 1973). The imbibed coat and large seed cavity in the tetraploid watermelon form a continuous wet layer around the embryo which the oxygen must transverse (Grange *et al.*, 2003). Seed treatments have enhanced germination in various field crops and vegetables. Combined application of ethephon and GA_{4-7} has improved germination in diploid watermelon (Nelson & Sharples, 1980). Germination and emergence of watermelon seeds also were improved by priming in salt solution (Sachs, 1977) and redrying after priming was a critical step for maintaining seed quality (Parera & Cantliffe, 1992). Seed coat removal in melon seeds improved germination at low water potential (Dunlap, 1988) and low temperature (Edelstein *et al.*, 1995). Mechanical weakening of the seed coat structure such as scarification, seed nicking and seed coat removal has been reported to successfully enhance germination of triploid watermelon seed (Duval & NeSmith, 2000, Grange *et al.*, 2000). Seed coat adherence to cotyledons in polyploid seeds is another problem in seedling emergence; however, seed orientation with the radicle end up decreased seed coat adherence (Maynard, 1989) but did not improve emergence.

There is need to add precision and achieve efficiency in tetraploid watermelon seed production. Hence the present study was laid out to evaluate different colchicine treatments for tetraploid plants induction and to identify tetraploid plants through different screening techniques. Effectiveness of seed alteration and chemical treatments on tetraploid watermelon seed germination and seedling stand were also determined.

MATERIALS AND METHODS

A total of 1000 seeds of diploid watermelon, 100 in each line, were germinated at 30°C in germinator for 48 hours, then planted in pots and placed in greenhouse. Ten seedlings of each line were grown as control and rest of the 90 seedlings of each cultivar were treated with different concentrations of colchicine, 0.2, 0.4 and 0.6%. Colchicine solution was injected in the meristem of seedlings at true leaves emergence stage twice daily for three consecutive days. Mortality

occurred in treated seedlings was recorded after 3 weeks of colchicine application. The efficacy of treatments to induce tetraploids was evaluated on the basis of chloroplast counts and flow cytometric analysis. The treated seedlings were evaluated for tetraploidy at 3-5 true leaves emergence. The first characterization of treated seedlings for tetraploidy was made by chloroplast counts in each side of stomata guard cells. On the basis of this evaluation, the putative tetraploid plants were transplanted in the greenhouse.

Flow cytometeric analysis was made using PA-1 (Partec, Germany) flow cytometer to reconfirm the ploidy of already screened out plants on the basis of chloroplast counts. Leaf tissue was chopped in a plastic Petri dish with 500 µl nucleus-isolation buffer and the suspension was passed through a 30 µm mesh filter and added 1 ml DNA staining solution for nuclear DNA content estimation. Comparison of tetraploid and diploid plants was also made by recording leaf area. Leaves from 7^{th}, 9^{th} and 11^{th} node of each plant were selected and measured under Li-Cor LI-3100 area meter. Flower size was recorded under Camscope with IT Pro Image Tracer software. Number of colpi in diploid and tetraploid pollen was counted under the light microscope at 400X magnification.

For germination, seeds of diploid and tetraploid lines were subjected as removed seed coat (naked embryo), nicked seed end (opposite to radicle end) and radicle end avoiding any damage to embryo. Intact seeds were treated as control. Seeds were placed in 9 cm petri dishes with two layers of filter paper, which was moistened with either 5 ml of distilled water. Seeds were incubated in darkness at a constant 30°C and germination was recorded on every 24 hours for 5 days. Seeds were considered to have germinated when the radicle protruded 3 mm from the seed coat. For seed coat adherence to cotyledons studies, seeds of both diploid and tetraploid watermelon were soaked for 2 hours in distilled water, placed on moist filter papers in 9 inch petri plates and incubated in germination chamber at 30°C for 48 hours. The germinated seeds were then planted either radicle side up or down in trays having 4x3 cm cells. Data regarding seed coat adherence and other abnormalities in seedlings was recorded during emergence of seedlings.

RESULTS AND DISCUSSION

Colchicine application delayed the emergence of true leaves about 2 weeks. In certain cases the meristem of the seedlings gets damaged and did not grow but some damaged meristems later on emerged adventitious shoots. Colchicine injury even appeared after emergence of 3-4 true leaves and symptomized as drying top of the stem. The increase in colchicine concentration increased the mortality rate in seedlings (Figure 1). It was minimum (20.5%) at 0.2% colchicines application. The variable response of lines for mortality percentage was noted. Overall the line NH_2 showed maximum mortality of seedlings (52%) followed by SS-11 (48.5%) while the lowest was in M_{174} (14.3%). Variable mortality rate after colchicine application has been reported by Suying *et al.* (1995) who applied colchicine after peeling-off leaflets and suggested that the injury increased the mortality rate than the control, without peeling-off. The variability in colchicine tolerance by different lines may be genotype dependent (Jaskani *et al*., 1996). Both increased levels and treatment duration of colchicine resulted in decreased seedlings survival rate (Koh, 2002). Chloroplast counts in each side of stomata guard cell pair showed that number of chloroplasts ranged from 5-7 and 10-12 in diploids and tetraploids, respectively (Figure 2; Figure 5c,d). In Moodeungsan watermelon the number of chloroplast in each side of guard cell pair were 12 in diploid and 22.8 in tetraploid (Koh, 2002). The number of chloroplasts seems to be variable in different lines. In certain cases this varied up to 8 or 13-15 chloroplasts in each guard cells pair. This might be due to mixoploid nature of leaf tissues (Koh, 2002) and was later on confirmed by flow cytometric analysis as chimeric plants. The results showed that chloroplast counts alone is not enough to screen out tetraploid seedlings because offsprings of such treated shoots were by no means all tetraploid (Kihara, 1951).

It was also observed that 0.2% colchicine induced maximum number of polyploids (60.7%) screened out on the basis of chloroplast counts (Figure 3). The increase in colchicine

concentration from 0.4-0.6% reduced the number of polyploids (49.8% and 37.8%, respectively). Similarly Suying *et al*. (1995) induced higher number of variants in watermelon using lower concentration of colchicine. Response of watermelon lines for polyploids induction showed that cultivar SS_7 produced maximum percentage of polyploids (74.4%) followed by NH_3 (69.5%) (Table 3). Interaction of treatments and lines indicated that SS_7 yielded maximum polyploids at 0.2 (83.1%) and 0.4% (75%) colchicine level. Rhodes and Zhang (1999) reported that tetraploids could be induced by applying aqueous colchicine solution to the growing apex of diploid seedlings or by soaking diploid seeds in colchicine solution prior to germination. However, the frequency of tetraploids from such treatments is less than 5% in most cases, and many plants were chimeras.

The diploid watermelon lines had genetically different sized leaves (Figure 4; Figure 5d,f). NH_3 had the largest (170.7 cm^2) and 920532 the smallest (99.5 cm^2) leaves among the diploid lines. Among the tetraploid lines, NH_1 yielded maximum leaf area (230.7 cm^2) followed by NH_3 (222.4 cm^2) and SS_1 (219.13 cm^2). Nwokeocha and Faluyi (1993) and Jaskani *et al*. (1996) examined tetraploids as vigorous with greater biomass and leaf breadth. Tetraploid flowers had larger size compared with diploids. Observation of pollen grains under microscope showed 3 and 4 colpi in diploids and tetraploids, respectively (Figure 5a & 5b). Rhodes and Zhang (1999) reported 3 colpi in diploids verses 4 in tetraploids.

Germination of the diploid lines was not affected by seed treatments, averaging 85% to 94% germination on all treatments (data not presented). However, tetraploid seeds showed significant differences of seed germination between treatments. Intact seeds (control) showed the lowest germination percentage (35.7%) as compared with other seed germination treatments. Seed coat removal improved the seed germination (84.3%) but was non-significant different with seed nicking at radicle end treatment which germinated 77.1% seeds. Seed coat of tetraploid seed has been reported thicker than that a diploid seed, which possibly limits the passage of water or gasses into the seed. Weakening the seed coat or the opening of small holes at the radicle end of the seed may facilitate movement of gasses into the seed (Nerson *et al*., 1985). Germination tests showed that when the seed coat was removed, higher germination percentages were obtained (Duval & NeSmith, 1999).

Seed coat adherence to cotyledons is a problem in tetraploid seeds that results in distorted and inconsistent seedling stand. Seed coat adherence was not affected by nicking; anyhow, nicking at seed end somewhat reduced the incidence of seed coat adherence (30%). A preliminary study was conducted to reduce seed coat adherence to cotyledons. The germinated seeds planted either radicle end up (90°) or down in potting media showed that diploid seeds did not affect by seed planting position and emerged almost all without any adhered seed coat to cotyledons. Tetraploid seeds planted in media with radicle end up emerged 74% seedlings without seed coat adherence and vice versa only 31%. Low germination and poor seedling emergence are major limiting factors in polyploid watermelon seeds (Andrus *et al*., 1971; Hall *et al*., 1989). Studies have implicated thicker seed coat or weak development of the embryo as causative factors (Kihara, 1951; Nerson *et al*., 1985). Maynard (1989) reported that adherence of seed coat to emerged cotyledons is troublesome problem, causing distorted seedlings and sometimes loss of plants. He also added that planting radicle end at a 45°-90° angle, reduced seed coat problem, but not eliminated.

CONCLUSIONS

In vivo application of colchicine to diploid watermelon lines for induction of tetraploids, describes that lower concentration was better for polyploids induction. Chloroplast count in stomata guard cells was quite good for initial screening of polyploids among large populations but flow cytometric analysis was the most authenticated. Tetraploid watermelon seed germination was difficult due to thick seed coat and underdeveloped embryo. Moreover, thick seed coat adhered to cotyledons and caused poor and less uniform seedling stand. Low seed germination in polyploids could be enhanced by seed nicking. We hope this research would serve as useful breeding method

for creating newer, improved triploids and propose to scale up these research findings for making commercial availability of triploid seed of watermelon in the country.

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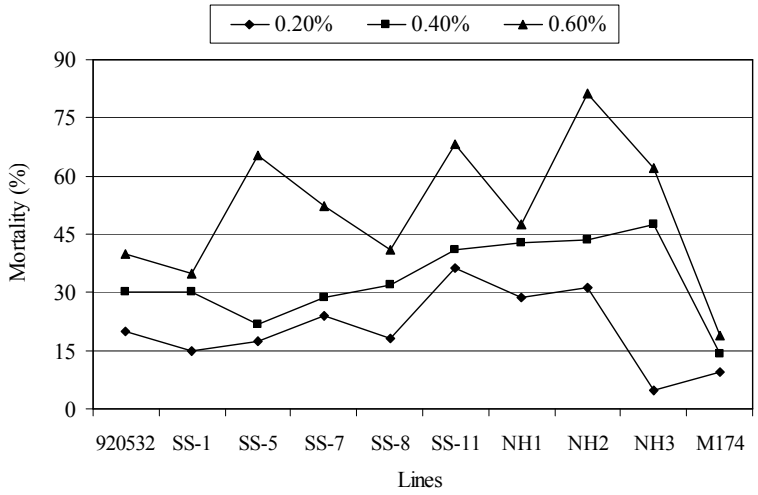

Figure 1: Mortality in colchicines treated water melon seedlings after 3 weeks of treatment

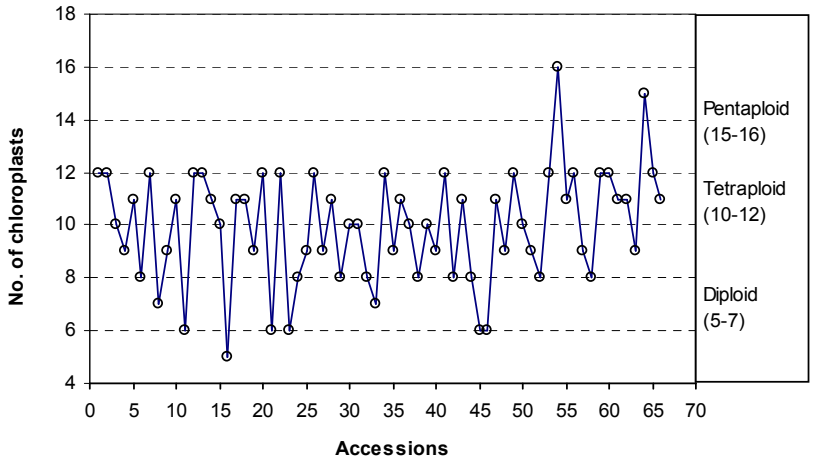

Figure 2: Classification of putative tetraploids on the basis of chloroplast counts in stomata guard cells in to diploids, tetraploids and mixoploids by flow cytometric analysis

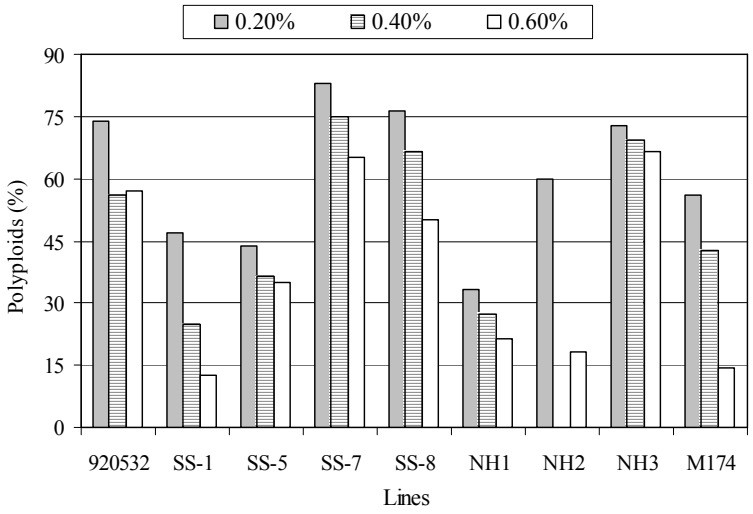

Figure 3: Induction of polyploids in various water melon lines after colchicines treatment in seedlings meristem

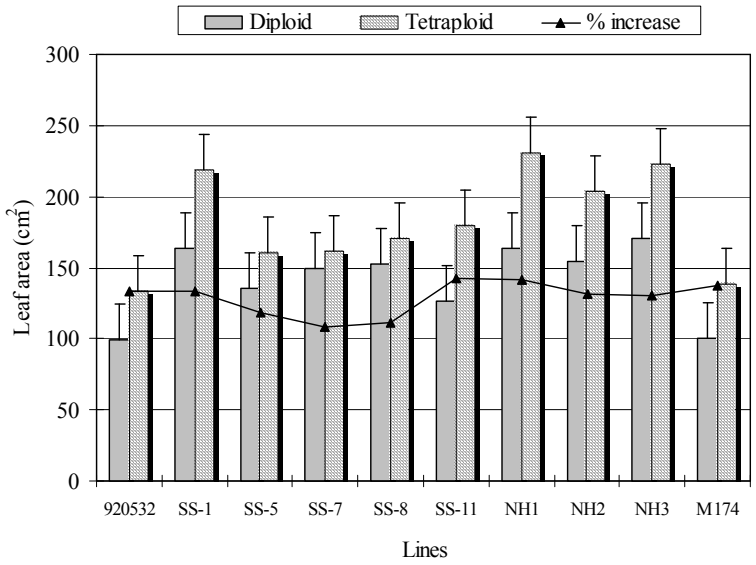

Figure 4: Increase in leaf area (cm^2) in tetraploid water melon as affected by colchicines treatment to diploids

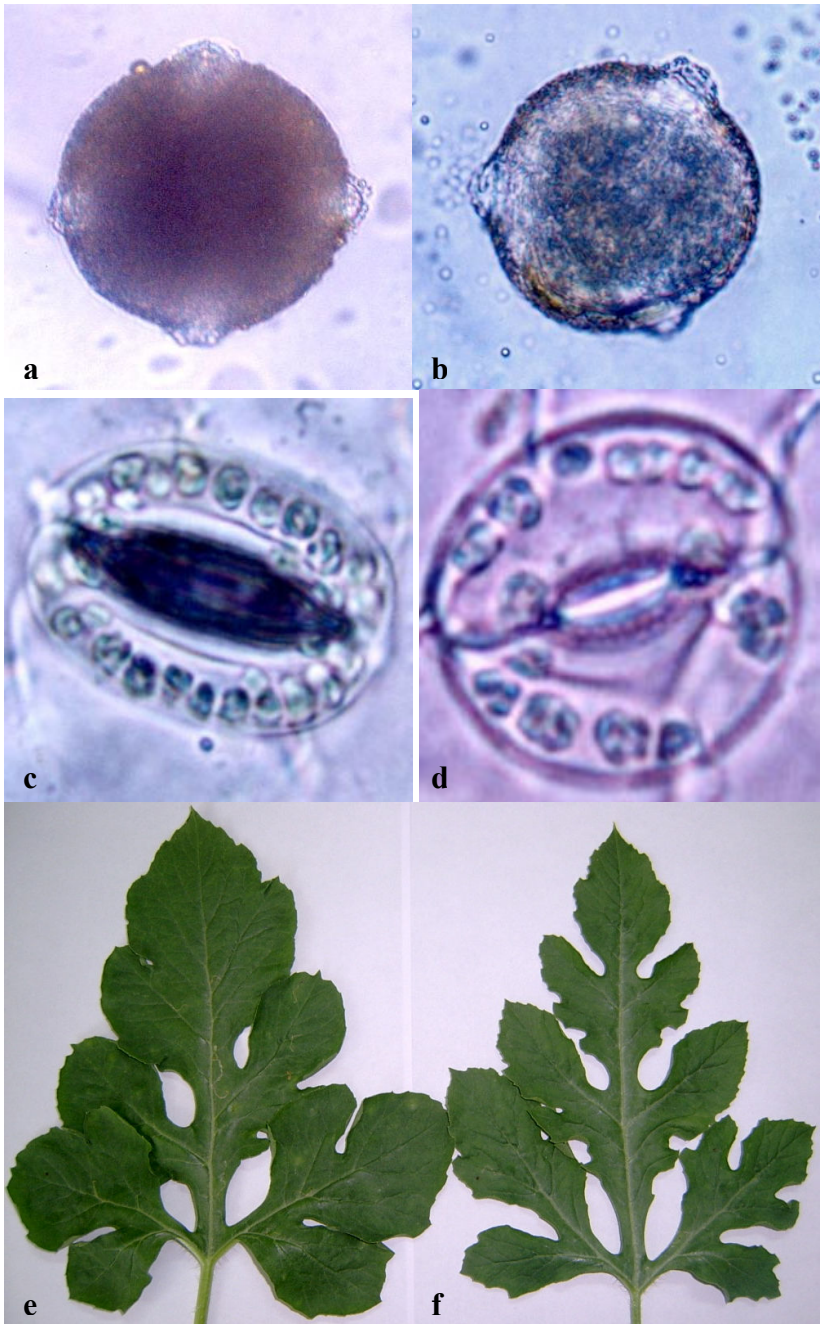

Figure 5: Variation in tertraploid and diploid watermelon plants. a) 4 colpi in tetraploid pollen; b) 3 colpi in diploid pollen; number of chloroplasts in stomata guard cells of c) tetraploid and d) diploid leaves; leaf area of e) tetraploid and f) diploid plants