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COLCHICINE INDUCED TETRAPLOIDS OF RADISH (*Raphanus sativus* L.)

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ABSTRACT

Radish (*Raphanus sativus* L., Brassicaceae) is an edible root crop cultivated all over Sri Lanka. So a few attempts have been made in Sri Lanka to improve crop for higher yield and wider adaptability. Therefore the present study was carried out to improve the Radish (*Raphanus sativus* L.); variety Beeralu through colchine. Petiole explants from *in vitro* regenerated plants were used to induce tetraploid by colchine chemical. The different concentrations of colchicine 0, 30, 60 and 120mgl^{-} were used. After 30 days interval colchicine treated petiole explants were transferred to the MS medium with 2.5mgl^{-1}BAP concentration without colchicine. Then effects of different time durations (5, 10, 15, 20 and 25 days) were examined using petiole explants in selected best colchine concentration (60mgl^{-1}) from the above trial. Cell samples of the root tips were used to identify chromosomes and stomata were observed from epidermal layer of leaves under a microscope (Axio Lab A1) and photos were taken with the associated apparatus to confirm ploidy level. Experiments were arranged according to a Completely Randomized Design (CRD) with five replicates and repeated four times with minimum of four replicates. Roots were induced MS medium with 0.25mgl^{-1} IBA. Highest percentage (25.27%) of tetraploid plantlets induction obtained from MS basal medium treated with 60mgl^{-1} colchicine for 20 days. Highest stomata length and width (467.54µm, 395.75µm) was observed from tetraploid plantlets while lowest leaf length (25.11cm), lowest root length(16.67cm) and highest leaf width (7.12cm) were reordered in acclimatized tetraploid plants.

***Key words*:** Beeralu variety, Colchine, Radish (*Raphanus sativus* L.), Tetraploid

INTRODUCTION

Polyploidy induction has been successfully applied in vegetables, ornamental and medicinal plants in order to gain lines revealing new agronomic characteristics. Polyploidy induction help to obtain significantly larger fruits, vegetables and flowers, long lasting flowers, seedless or fewer seed fruits, flowers with greater number of petals pest resistance and physical stress tolerance crops (Predieri, 2001). These effects can differ according to species, degree of heterozygosity and ploidy level (Haiyan *et al*., 2013).

Polyploidy plants have been obtained from both *ex vitro* and *in vitro* approaches (Praca *et al*., 2009). Colchicine treatment is *ex vitro* approach which had been widely used to induce polyploidy in some fruit crops such as grape (Derman, 1960), banana (Van *et al*., 1996) and apple (Liu *et al*., 2001).

Radish (*Raphanus sativus* L.; family Brassicaceae) is an important root crop cultivated throughout Asia (MiAe Cho *et al*., 2008). Radish is one of the vegetables that can be grown in all agro ecological regions in Sri Lanka throughout the year if adequate moisture is available The edible root with different shapes which can be used to both culinary and medicinal properties. Major genetic improvement of radish has been achieved by conventional plant breeding methods, such as crossing in Sri Lanka as well as world. However, these methods are time and labor consuming. Moreover there is limited information on the

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polyploidy of radish both overseas and Sri Lankan variety. Likewise polyploidization of Brassicaceae family crops have low harvesting period than other families. Therefore the aim of this research was to establish polyploidy induction protocol for Radish (*Raphanus sativus* L.) Var. Beeralu Rabu by colchine.

MATERIALS AND METHODS

In vitro grown diploid plants were regenerated on MS basal medium containing 2.5mgl^{-1} BAP and 0.01mgl^{-1} NAA. Petioles of the plantlets were used as explant all over the experiment (Caperta *et al*., 2006).

Polyploidy induction

Concentrations of colchicine

In vitro petiole explants (5-10mm) excised from the above regeneration medium introduced to MS basal medium containing different concentrations of filter sterilized colchicine (0,30, 60, 120mgl^{-1}). After 30 days interval colchicine treated petiole explants were transferred to MS basal medium with 2.5mgl^{-1} BAP without colchicine. Five explants were cultured in one bottle.

Duration of treating of colchicine

From the results of above experiment, select the best colchicine concentration to double the chromosome number and conduct the following experiments to investigate the effect of treatment duration to produce the polyploids.
Prepared 30 bottles of MS medium with 2.5mgl^{-1} BAP + 0.01mgl^{-1} NAA + 60mgl^{-1} colchicine and transferred 1/5 of the explants from day 5,10,15,20,25 and 30 to fresh medium with the same composition of hormones without colchicine and kept back30 days.

Determination of ploidy level of Radish

Fifty regenerated plants were randomly selected and from each plant all actively growing root tips and leaf tips about 5-10 mm in length were excised. These root tips and leaf tips were washed with tap water for removing the residues of the medium, and then pre-treated with 0.01% colchicine solution at room temperature for 2hours. Then they were washed with tap water and transferred to fixing in Carnoy's solution containing acetic acid: ethanol (95-100%) (1: 3, v/v) for at least 18 hours. The fixed root tips and leaf tips were then hydrolyzed in 1 N HCl for 03min at 65^{0}C. After hydrolysis, root tips and leaf tips were rinsed with distilled water for 10min and cut to obtain shorter root tips and leaf tips about 1.5mm. These prepared root tips were then placed on slid glass, stained with one drop of carbol fuchsin solution for 1-2minutes, squashed under cover glass and cell samples of the root tips were observed for chromosomes under a microscope (Axio Lab A1), and photos were taken with the associated apparatus.

A plant with all the root tip cells and leaf tips cells of *R. sativus* showing 18 chromosomes was determined as diploid, a plant with some cells showing 18 and the other cells showing 36 chromosomes was determined as chimera, and a plant with all the cells showing 36 chromosomes was determined as tetraploid.

Stomata analysis

For stomata analysis, a few pieces of epidermal layer were torn from the abaxial side of relatively mature leaves of tetraploid, chimeric, diploid plants separately. These epidermal layers were then mounted on glass slide with one drop of distilled water and a cover glass for measuring sizes and taking photos of stomata under a microscope.

Root induction of shoots

Healthy and robust regenerated shoots longer than 1.5 cm were cut from the mother tissues and cultured to MS medium with 0.25mgl^{-1} IBA for the initiation of roots and further growth of the intact regenerated plantlets. To verify the ploidy level of the treated plants, counted the number of chromosomes using young root tips.

Acclimatization and transferring of plants

After 60 days well elongated plantlets removed

from the media and washed them well, but gently with water to get rid of any race of gelling agent on the plantlets, especially on the root to avoid possible contaminations during the acclimatization process. These plants were then placed in the wide mouth glass bottles of about 250ml inner space, which contained water, just to cover the root system of the plants. Relative humidity in the bottles was gradually reduced by opening the closures of the bottles till they reach the conditions similar to outside environment.

Observation of agronomic characteristics

Acclimatized tetraploid and diploid plantlets of *R. sativus* were transplanted to the pots containing soil in the experimental field in Faculty of Agriculture, University of Ruhuna and plants were selected for further identification of agronomic characteristics. After care operations such as watering, fertilizer and insecticide application were done as necessary.

Collection of data

At all stages, percentage of regeneration, number of shoot per explant, time taken for the regeneration, number of tetraploid, diploid and chimera plants were calculated for the comparison of the effect of colchicine on doubling the chromosome number of *R. sativus.*

All experiments were done under CRD design and data were analyzed by SAS statistical tool.

RESULTS AND DISCUSSION

Shoot regeneration ability and growth of buds were delayed on colchicine treated media than non treated media (Table 1). Hypocotyl explants were taken long time to regenerate shoots on colchicine treated medium than col-

Figure 1. Cultures of hypocotyl explants on regeneration medium containing 2.5mgl^{-1}BAP and 0.01mgl^{-1} NAA (after 30 days) a) without colchicines, b) containing 30mgl^{-1} colchicine 2.5mgl^{-1} BAP, 0.01mgl^{-1} NAA, c) containing 60mgl^{-1} colchicine 2.5mgl^{-1} BAP, 0.01mgl^{-1} NAA, d) containing 90mgl^{-1} colchicine 2.5mgl^{-1} BAP, 0.01mgl^{-1} NAA

Table 1. Effects of various colchicine concentrations on regeneration of petiol explant of radish var Beeralu

Colchicine concentration (mgl^{-1})	% of shoot regeneration	Number of shoots per bottle	Number of days taken to regenerate
0 (control)	98.2^{a*}	8.1^{a}	5.2^{c}
30	64.5^{b}	5.9^{b}	10.6^{b}
60	49.3^{c}	4.0^{c}	20.5^{a}
90	00.0^{d}	0.0^{d}	00.0^{d}
120	00.0^{d}	0.0^{d}	00.0^{d}

*Data in the same column followed by different letters are significantly different by Duncan's test at 95% level

chicine non treated medium. Therefore regeneration progress was delayed by the colchicine. After 30days of experiment, all untreated explants had indicated significantly higher number of shoots than colchicine treated explants. Furthermore shorter and thick shoots were obtained on colchicine treated media (Figure 1). Regeneration of explants were prevented completely in the highest concentration of colchicine (120mgl^{-1}).

When colchicine concentrations were increased**,** the lethal percentages of explants were increased. Non growing, brown buds were considered to be dead explant. Explants were survived on the 30mgl^{-1}, and 60mgl^{-1} colchicine treated media (Table 1). However, all of the explants treated with 90mgl^{-1} and 120mgl^{-1} colchicine were died.

Similarly, as stated by (Caperta *et al*., 2006) negative effects on shoot regeneration and development were detected in medium with high colchicine concentrations in banana and tetraploids were induced 8mM colchicine for 96 hours in tomato plants (Caperta *et al*., 2006).

Hypocotyl explants which were treated with various concentrations of colchicine for 12 days, and the effects of colchicine on inducing chromosome doubling were evaluated by counting the chromosome numbers in the root tip cells of the plants regenerated from the colchicine treated hypocotyl after 30days (Fig 2). The plants with both diploid and tetraploid cells in the same plant were regarded as chimera plants and all cell appeared 36 chromosomes, characterized as tetraploid plants.

The effects of colchicine at different concentrations on doubling chromosome number were summarized in Table 2.

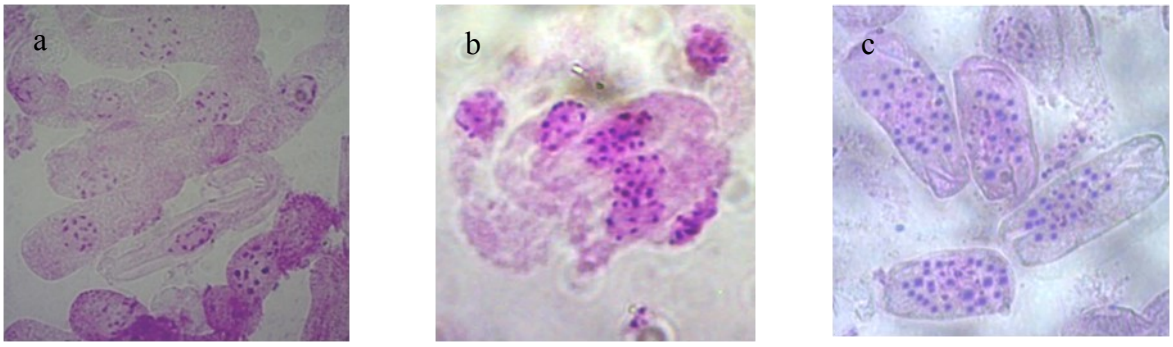

Figure 2. Plant cells a) diploid (2n=18); b) chimera c) tetraploid (2n=36) (These three photos are all original, they were taken under the same microscopic conditions; Microscope Axio Lab A1)

Table 2. Effects of various colchicine concentrations on regeneration of petiol explant of radish var Beeralu

Colchicine concentration (mgl^{-1})	No. plants sampled	No. root tips sampled	No. cells observed	No. cells with only 2x chromosome (Diploid)	No. cells with only 4x and 2x chromosome (Chimera)	No. cells with only 4x chromosome (Tetraploid)	Tetraploid percentage
30	50	50	750	654	68	28	3.73
60	50	50	752	531	46	175	22.96
90	00	00	000	0000	000	000	00
120	00	00	000	0000	000	000	00

A number of explants can be used as starting material to induce polyploidy. Young, actively growing explants containing a meristem usually give best results. For example, success has been found using germinating seeds, *ex vitro* shoots, roots, embryogenic callus, non embryogenic callus, nodal segments, cotyledons, and hypocotyls (Glendon *et al*., 2008). During micro propagation of *Radish*, we observed that only the hypocotyl region of seedlings was capable of regenerating adventitious shoots, probably because they contain the shoot apical meristem and thus would make suitable explants for ploidy induction (Glendon *et al*., 2008). Therefore hypocotyl explants were used in this experiment and in addition because of hypocotyl was the selected best regenerated explants in above experiment.

The highest chromosome doubling was induced by $60mgl^{-1}$ colchicine treated media. Therefore explants were inoculated on media supplemented with $60mgl^{-1}$ colchicine for various durations in this experiment. Highest regeneration frequency was occurred in lowest days (5days) in colchicine treated explants while lowest regeneration percentage was occurred in highest days (25days) in colchicine treated explants. No shoots survived from the highest treatment duration (25 days) and the all explants died (100% lethality) (Table 3). When increasing the treatment duration**,** the percentages of regeneration and number of regenerated shoots per explant were reduced simultaneously and time taken to regenerate was decreased.

Table 3. Regeneration frequency, Number of shoots observed and days taken to regenerate when colchicines treated explants of Radish (*Raphanus sativus* L.), were exposed to indicated treatment durations

Treatment duration (days) in $60mgl^{-1}$ colchicine	Regeneration frequency %	Number of shoots per bottle	Number of days taken to regenerate
05	79.0^{a*}	9.8^{a}	20^{d}
10	62.0^{b}	6.6^{b}	30^{c}
15	46.0^{c}	2.3^{c}	38^{b}
20	16.7^{d}	1.3^{d}	49^{a}
25	00.0^{e}	0.0^{e}	00^{e}

*Data in the same column followed by different letters are significantly different by Duncan's test at 95% level

Table 4. Comparison on chromosome doubling effects of different colchicine treating durations

Colchicine duration (days)	No. plants sampled	No. root tips sampled	No. cells observed	No. cells with only 2x chromosomes (Diploid)	No. cells with 2x and 4x chromosomes (Chimera)	No. cells with only 4x chromosomes (Trtaploid)	Tetraploid percentage
5	50	50	723	654	52	17	2.35
10	50	50	741	621	41	79	10.66
15	50	50	738	598	39	101	13.65
20	50	50	732	519	28	185	25.27

Chromosome number of the root tip cells was counted from root tips in regenerated shoots in grown plantlets. The highest percentage (25.27%) of tetraploid plantlets induction was occurred in the regeneration medium treated with 60mgl^{-1} colchicine for 20 days while lowest tetraploid percentage (2.35%) was observed in treatment for 5 days (Table 4). It is possible to artificially create polyploid plants by interfering with cell division. A number of natural and synthetic compounds can be used and are either applied to plants *ex vitro* or *in vitro*. Colchicine is naturally occurring, and it was initially thought that it promoted polyploidy induction by disrupting spindle formation and preventing nuclear and cell division (Glendon *et al*., 2008). Recently, however, Caperta *et al*., 2006 showed that spindle disruption alone is insufficient for the production of polyploid cells. Low concentrations (0.5mM) of colchicine inhibited microtubule formation in all phases of the cell cycle but resulted in abnormalities, including reduced viability from irregular shaped nuclei and micronuclei. In contrast, treatment with high concentrations (5mM) of colchicine induced microtubule polymerization to form new structures in c-metaphase cells. These new structures are thought to aid reconstitution of polyploid nuclei and subsequent re-entry into the cell cycle (Caperta *et al*., 2006). Effectiveness of these colchine *in vitro* depends highly on the concentration applied, duration of treatment, type of explant, and the penetration of the compound (Glendon *et al*., 2008). Colchicine has been effectively used in the concentration range 0.25µM to 38,000µM (Glendon *et al*.,2008).

Colchicine concentration and treatment duration of colchicine significantly affected the chromosome doubling in this study. The lower number of plantlets was obtained in the highest colchicine concentration and highest duration. According to strong toxic effect of colchicine in plant cells are reason for gain lower number of plantlets. However colchicine causes side effects such as sterility, abnormal growth and morphology, chromosome losses or rearrangements and gene mutation in many species (Luckett,1989). The tetraploids that obtained, could be used in further breeding work. This study demonstrated for the first time that colchicine is effective producing tetraploids in radish. Low colchicine concentrations with short time duration were used to induce tetraploid in preliminary experiments (Song *et al*., 1997). However those attempts were not effective, because tetraploid were not produced except chimeric plants. Therefore on the bases of the preliminary experimental results, the plant materials were treated with low concentrations of colchicine for longer duration in the present study. However there were no any attempts to produce tetraploid of radish previously.

Moreover long duration with low colchicine concentrations were much less attempted while high concentrations of colchicine for long durations were much attempted Tetraploids were obtained by Chakraborti *et*

Table 5. Comparison of stomata sizes among tetraploid and diploid plants of radish var Beer-alu

Ploidy level of plant	Stomata length (µm)	Stomata width (µm)
Tetraploid	467.54[a*]	395.75[a]
Chimera	401.13[b]	324.92[b]
Diploid	351.32[c]	281.12[c]

*Data in the same column followed by different letters are significantly different by Duncan's test at 95% level

al., (1998) in mulberry with 1000mgl^{-1} colchicine for 28 days and moreover less tetraploid percentage was obtained in annatto with 10mgl^{-1} colchicine for 15 days by Haiyan *et al* (2013). In the present study, we found that colchicine at 60mgl^{-1} was appropriate for induction of chromosome doubling, and longer duration (25days) of colchicine treatment was necessary for obtaining completely doubled chromosome (tetraploid plantlets) from diploid explants

Chimeric plants were produced in 30mgl^{-1} and 60mgl^{-1} and every time durations in colchicine treated media which were tested in this experiment. High production of chimeras in regenerated plants from colchicine treatments have been reported in many cases, such as in mulberry (Petersen *et al*., 2003) and in *Miscanthus sinensis* (Eeckhaut *et al*., 2004) and chimeric plants were by product of the production of tetraploids in chemical induction of tetraploids. Chimera plants were displayed beneficial characters according to Hamil *et al* (1992) as well.

Chromosome counting in metaphasic cells is considered a consistent method for determining ploidy levels of plants. Therefore that method was used for the present study. However most reliable, practical and rapid method to determine ploidy level is flow cytometric method (Zhang *et al*., 2008). Nevertheless that method could not be used for the present study since relevant facilities were not established in the research laboratory.

The morphological features of polyploidy plants were evaluated to determine whether they could be used to identify reputed tetraploids. There was prominent morphologically different of plant leaves could be identified clearly in *in vitro* and *in vivo*. In most cases, the leaves of tetraploids appeared thick and short and stomata sizes were significantly different (Figure 3).

Figure 3. *in vitro* plants a) tetraploid plantlets of radish var Beeralu (right side) b) diploid plantlets of radish var Beeralu

Ploidy manipulation offers some benefits for horticultural, pharmaceutical, and agricultural improvement of plants. From a horticultural perspective, polyploidy plants often have a shorter height, and the larger, thicker leaves and bigger flowers are more attractive to consumers (Glendon *et al*., 2008).

As stated by Chen *et al.,* (1979) polyploids are usually superior than diploids when comparing to genetic adaptability and resistant to environmental stress. In horticulture, Polyploids are more widely used for their improved ornamental characteristics such as larger flowers and larger fruit in horticultural industry (Gao *et al*., 1996).

Table 6. Comparison of morphological characters among tetraploid, chimera and diploid

Morphological characters	Diploid	Chimera	Tetraploid
Leaf length (cm)	28.21^{a}	26.36^{b}	25.11^{b}
Leaf width (cm)	6.44^{b}	5.93^{c}	7.12^{a}
Root length (cm)	15.32^{a}	14.98^{b}	14.67^{b}
Root width (cm)	5.87^{a}	5.21^{b}	5.71^{a}

*Data in the same column followed by different letters are significantly different by Duncan's test at 95% level

Stomata were observed in *in vitro* grown diploid, chimera and tetraploid plants which were confirmed by chromosome number. Differences of sizes of stomata were identified through microscopic observations. Size of stomata on leaves varied largely different within same leaf; however sizes of stomata were significantly different between diploid, chimera and tetraploid plants (Table 5). The bigger size of stomata was obtained from the tetraploid plants than diploid plants (Figure 4).

Stomata analysis is one of reliable method to determine ploidy level (Carvalho *et al*., 2005). Therefore the measurement of stomata length could be an effective way to select tetraploid and diploid plants of radish as well as other plants. Length and width of stomata of tetraploid were almost bigger than observed diploid plants of radish.

Stomata of induced polyploids are usually larger than diploid in same plant and it is a simple and accurate method to identify polyploidy level (Glendon *et al*., 2008). And it is rapid, inexpensive, non-destructive, does non-sophisticated, and fairly high accurate method (up to 90% in some cases; (Glendon *et al*., 2008).

Root initiation was observed 25 days after transfer to rooting medium (0.25mgl^{-1}IBA) in all diploid and tetraploid plants from different concentrations of colchicine. Longer roots were found in diploid plants than in same age tetraploid ones. Tetraploid plants were obtained thicker roots than diploid plants (Figure 5).

Figure 5. Roots of regenerated diploid plantlets of radish var. Beeralu (Left), roots of regenerated tetraploid plantlets radish var. Beeralu (right side)

Healthy well grown tetraploids and diploids were weaned from culture and transferred to soil. Tetraploid plants grew normally in the field (Figure 6). There were no significant differences in harvesting time between diploid with tetraploids. Conferring to the morphological data root yield of tetraploid radish var. Beeralu lower than diploid radish in the same variety. But in tetraploids leaf yield was higher than the diploid plants (Table 6) (Figure 7).

Figure 6. Acclimatized radish var. Beeralu plants in the field

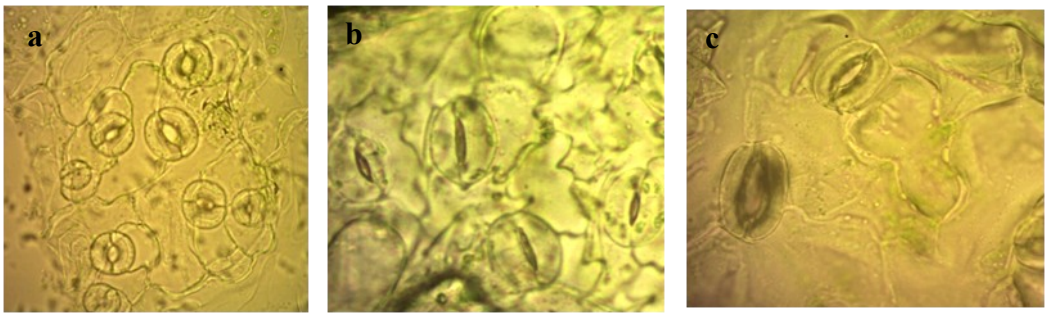

Figure 4. Stomata of *in vitro* grown *R. sativus*; a) diploid plant; b) chimera plant; c) tetraploid plant

Highest leaf length was obtained by diploid plant and leaf lengths of chimera and tetraploid plants were not significantly different. Furthermore highest leaf width, root lengths and root width were achieved by tetraploid plant.

Tetraploid plants displayed retarded growth in the present study than diploid plants. According to Chakraborti *et al., (*1998), retarded growth occurred in colchicine treated explants may be due to a physiological disturbance caused by colchicine, resulting in a reduced rate of cell division.

Polyploid plants were gained special characters such as high yield, high product quality and both biotic and abiotic stresses tolerance. Furthermore polyploidy can be used as a bridge for gene transferring between species which have different ploidy level and polyploidy often results in reduced fertility due to meiotic errors, allowing the production of seedless varieties. And also the genome doubling in a newly formed sterile hybrid allows the restoration of its fertility (Specialty seeds).

Correspondingly polyploidy plants have drawbacks than diploid plants such as infertility, low dry matter content, than diploid plants (Sattler *et al*., 2016). That may be the reason for yield of radish var. Beeralu in the present study lower than diploid plants in same variety.

Diploid Chimera Tetraploid

Figure 7. Morphology of tetraploid, chimera and diploid plants of *Rapunus sativus*

Moreover mutation frequency is excessive than the corresponding diploid cultivar due to the large genome. This approach has been used in mutation breeding of chimenes spp. (nut orchids) by first forming auto tetraploids through colchicine treatment and 20-40 times higher than corresponding diploid plants (Broertjes., 1976).

CONCLUSION

The highest percentage (25.27%) of tetraploid plantlets induction gained in the regeneration medium (MS medium with 2.5mgl^{-1}BAP) treated with 60mgl^{-1} colchicine for 20 days from radish var Beeralu. Largest size stomata were gained from tertaploid plants while deliberate morphological characters; highest leaf width, root lengths and root width were achieved by diploid plants. Nevertheless yield of tertaploid plants is lower than the diploid plants and nutrition content was not analyzed in the present study. Even though yield is low, nutrition content may be high. Therefore the present study can be carried out further to that approach. Same technology can be applied to improve existing crops due to shorter time was spent than other hybridization methods.

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