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In Vitro Chromosome Doubling in Korarima [*Aframomum corrorima* (Braun) P.C.M. Jansen] Using Colchicine and Oryzalin

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ABSTRACT

Ploidy manipulation holds high promise for the improvement of korarima (*Aframomum corrorima*) plants with altered morphology, as well as of potentially altered essential oil yields and oil composition. The present work aimed to establish a protocol for *in vitro* polyploid induction of korarima. Nodal explants 2 mm long were cultured in liquid media with 0, 125, 250, 375 or 500 µM colchicine or 10, 20, 40, 60 or 80 µM oryzalin for 7 d. The lowest concentration was the most effective for polyploid induction with both colchicine and oryzalin. Teteraploid plants were obtained only from shoots regenerated from explants treated with 125 µM colchicine and 10 µM oryzalin. Oryzalin was thus confirmed to be effective at a lower concentration than colchicine.

Keywords: polyploid, mixoploid, cytochimera, indexing

INTRODUCTION

Korarima [*Aframomum corrorima* (Braun) P.C.M. Jansen], an indigenous and endangered species of Ethiopia, is a spice and medicinal plant of economic importance (Jansen, 1981; Sebsebe, 1993). The crop has a relatively wider adaptation, higher productivity and also better tolerance to moisture stress than its renowned close relative, Indian cardamom (*Elettaria cardamomum*) (Jansen, 1981; Purseglove *et al*., 1981) and is less peppery or pungent than seeds of other African species, such as *Aframomum melegueta* (Grains of Paradise); this is mainly attributed to the high ratio of 1,8-cineol to terpynyl acetate (38:11), which is the primary cause for its typical "Nutmeg cardamom" odor (Jansen, 2002).

According to Osborn *et al*. (2003), many studies reveal polyploidy not to be a state of simple genome duplication, but a genetic manipulation that could result in a whole spectrum of molecular and physiological modifications. Such induced genetic variation arising from polyploidization is ascribed to the overall changes attained in dosage-regulated gene expression, altered regulatory interactions, as well as rapid genetic and epigenetic changes. For genes having an allele dosage effect or multiple alleles, polyploidy could also increase the potential genetic variation in their expression levels (Vaughan *et al*., 2007). Accordingly, polyploidy often produces immediate and dramatic changes in structural, biochemical, and physiological elements, which might influence their photosynthetic rates (Ojiewo *et al*., 2007).

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A beneficial effect of increasing the chromosome number is that it may sometimes enhance the expression and concentration of certain secondary metabolites and defense chemicals, which are mostly related to the increase in the size of the oil-producing organs (Jones *et al*., 2008). As a result, polyploidization is considered a beneficial breeding technique to enhance the production potentials of plant secondary metabolites, especially the yield and composition of essential oils (De Jesus-Gonzalez and Weathers, 2003; Nilanthi *et al*., 2009). This was primarily ascribed to the genomic multiplication which would confer enhanced production and qualitative improvement or both in the biochemical profile of the associated secondary metabolites by De Jesus-Gonzalez and Weathers (2003) who observed boosted contents of artemisinin in tetraploid *Artemisia annua* compared to the diploid counterpart. Maruška *et al*. (2010) also obtained an increase in both the quantitative and qualitative compositions of secondary metabolites in a number of polyploid medicinal plant species, such as three lobe beggar ticks (*Bidens tripartita* L.), fenugreek (*Trigonella foenum-graecum* L.), milk thistle (*Silybum marianum* L.) and marigold (*Calendula officinalis* L.). A relative increase from the diploid in the secondary metabolite production per unit dry weight of the autopolyploid was also reported in different medicinal plant species including *Atropa*, *Camellia*, *Hyocyamus* and *Solanum* (Dhawan and Lavania, 1996). However, the negative impacts on productivity of the natural products were observed in some other plant species, such as *Datura* and *Mentha* (Dhawan and Lavania, 1996).

As reported by Nilanthi *et al*. (2009), chromosome doubling may also change the compositions of secondary metabolites produced by a particular plant species. The changes observed in the metabolic profile of autopolyploid plants due to mere duplication of their basic genome have been ascribed to the associated disturbances created to the intrinsic metabolic mechanisms that regulate biosynthesis of those specific compounds. On the other hand, Dhawan and Lavania (1996) have ascribed these to the resulting multiplicity of allelic combinations in the polyploid that could have offered the advantage of producing hybrid enzymes and enzymatic diversity, which in turn could provide greater versatility and homeostasis. In accordance with this, *in vitro* polyploidization brought about considerable variations in the chemical constituents, morphology or agronomic characters or both of cardamom, which is one of the closest relatives of korarima (Sudharshan, 1989).

There are several types of chemical inducer used in polyploid induction, including colchicine, oryzalin (3,5-dinitro-N4, N4-dipropylsulfanilamide), trifluralin, amiprophos-methyl, and N_2O. All these chemicals are known to bind to the tubulin dimers preventing the formation of microtubules, and hence spindle fibers, during cell division (Hansen and Andersen, 1996). Thereby, they prevent the migration of daughter chromosomes to opposite poles. This process is known as mitotic slippage, which leads to the creation of a polyploid cell (Petersen *et al*., 2003. Among the antimitotic chemicals, colchicine and oryzalin are by far the most common used in various plant species (Ojiewo *et al*., 2007; Dhooghe *et al.*, 2011).

Recent studies in a number of plant species suggested that oryzalin shows strong binding ability to plant tubulins, which in turn makes it much more effective than colchicine for plant polyploidization (Morejohn *et al*., 1987; Kermani *et al*., 2003; Nadler, 2009). According to Morejohn *et al*. (1987), several reports attested oryzalin to be much more effective in polyploid induction at micromolar-level concentrations, in contrast to that of colchicine, which is used at millimolar concentrations. Therefore, oryzalin is gaining wider acceptance as a means of obtaining solid polyploids in various plant species instead of using colchicine (van Tuyl *et al*., 1992; van Duren *et al*., 1996; Fong, 2008; Kanchanapoom and Koarapatchaikul, 2012). It is also relatively

cheaper, safer to handle and produces minimal proportions of chimeras (Kermani *et al*., 2003; Fong, 2008).

The *in vitro* culture environment is more beneficial for polyploidy induction than *in vivo* conditions as it can promote synchronized cell divisions (Ross *et al*., 2000; Predieri, 2001). It thereby increases the induction efficiency and hence minimizes the occurrence of mixoploids/ cytochimera (Chakraborti *et al*., 1998). As reviewed by Jones *et al*. (2008), the success of the *in vitro* application methods over the earlier *in vivo* techniques could somehow be associated with the thinner cuticle (which results from the constant high humidity conditions of tissue culture) and the degree of bathing and hence maximum exposure of the explants to the solution in the course of treatment. In addition, the technique eases the need for monitoring of the successive processes in the course of polyploid production, as well as subsequent regeneration of polyploid plants through tissue culture (Fong, 2008).

Ploidy determination commonly relies on chromosome counting in mitotic cells of root-tips and nuclear DNA content analysis; however, simple methods for the preliminary screening of putative polyploids making use of some morphological characters are highly beneficial to reduce the time and labor allotted for chromosome counts. Characteristics, such as leaf size, stomata cell sizes, number of chloroplasts in the guard cells, stomatal density, pollen grain diameter and stem thickness are among the prominent morphological features that may reveal a considerable increase as a consequence of polyploidization. Those morphological indicators associated with stomatal characters are widely used for preliminary segregation of putative polyploids, as they are relatively more precise and easy to evaluate than other indicators (Sudharshan, 1989; Nilanthi *et al*., 2009; Grouh *et al*., 2011; Rêgo *et al*., 2011).

Different techniques of polyploid induction have been used with success in several members of the family Zingiberaceae (Sudharshan, 1989; van Duren *et al*., 1996; Adaniya and Shirai, 2001; Sakhanokho *et al*., 2009; Kanchanapoom and Koarapatchaikul, 2012). However to date, there have been no reports on the determination of the chromosome number of korarima and no attempt has so far been made to test the possibility of *in vitro* polyploidization for the genus *Aframomum* in general and korarima in particular, to widen the prevailing narrow genetic pool. Therefore, the present study was undertaken to establish an efficient methodology for the *in vitro* induction of polyploidy using both colchicine and oryzalin in korarima. In addition, the potential of indexing with different stomatal characters was also evaluated with regard to their benefits for the preliminary screening of putative korarima polyploids.

MATERIALS AND METHODS

All activities of stock plant maintenance, explant collection, sterilization, culture initiation and shoot multiplication were performed following previously outlined tissue culture procedures (Tefera and Wannakrairoj, 2004). Accordingly, shoot multiplication was carried out using Murashige and Skoog (1962) (MS) basal medium supplemented with 5% coconut water, 3 $mg.L^{-1}$ benzyl adenine (BA), 0.5 $mg.L^{-1}$ thiadiazuron, 3% sucrose, and 0.7% agar-agar (henceforth referred to as the culture medium).

Actively growing *in vitro* shoots of 'Jimma local' korarima were collected from the multiplication medium to be used as explants in this study. Initially, stock solutions of both microtubule depolymerising agents were prepared in dimethyl sulfoxide (DMSO), at concentrations of 0.25 μM and 4,000 μM for colchicine and oryzalin, respectively. Five levels of colchicine (0, 125, 250, 375 and 500 μM) (Sigma-Aldrich; St. Louis, MO, USA) and oryzalin (10, 20, 40, 60 and 80 μM) (British Greyhound Chromatography and Allied Chemicals; Birkenhead, UK) were evaluated in the experiment. In all treatments,

including the control, the proportion of DMSO added to the culture medium was manipulated so that its final concentration in the respective medium was maintained at exactly 2%. In this particular study, DMSO was selected and used as it is a suitable solvent and sterilizing agent in addition to its potential to enhance penetration of the anti-microtubule agents through the cell walls.

Liquid culture medium was prepared and sterilized in 10 flasks and allowed to cool to 60 ^{o}C under aseptic conditions prior to adding solutions of the antimitotic agents. Then, the volume of the culture medium in each of the flasks was adjusted to 600 mL after the addition of the necessary quantities of microtubule depolymerising chemicals and DMSO supplement under aseptic conditions. A sample of 20 mL of the prepared medium was dispensed into 125 mL Erlenmeyer flasks in a laminar airflow cabinet. Shoot explants of korarima were trimmed into leafless 10–15 mm nodal sections and cultured. Flasks were then kept on a rotary shaker (120 rpm) in the dark for 7 d. Each treatment contained 10 replications, whereby three explants were used per replication.

After 7 d, the microshoots were collected and rinsed three times in sterilized distilled water. These shoots were then recorded and transferred singly into a plant growth regulator (PGR)-free liquid MS medium devoid of the anti-mitotic agents and kept on the shaker (100 rpm) for 2 wk. Later, the surviving shoots from each treatment were recorded and subcultured onto a semi-solid shoot proliferation medium (Tefera and Wannakrairoj, 2004) for another 8 wk to maximize the number of regenerants from each treatment. The proliferated shoots from each treatment were further sub-cultured onto a semi-solid MS basal medium supplemented with 5% coconut water, 3 mg.L^{-1} BA and 1 mg.L^{-1} kinetin for elongation and production of normal shoots. Finally, individual shoots from each treatment were sub-cultured twice at monthly intervals onto a fresh PGR-free MS medium for rooting and plantlet production. At the end of the second sub-culture on a PGR-free medium, initial categorization of plantlets was carried out based on their morphology. The main morphological characters used to segregate the surviving plants for subsequent tests were the vigor of the regenerated plantlets, as well as the size and color intensity of their leaves, using the diploid controls as a baseline. These plantlets were further categorized using stomatal characters, such as the stomatal density, stomatal length and stomatal index, following the procedures described by other researchers (van Duren *et al*., 1996; Huang *et al*., 2010). Subsequently, chromosome counting was carried out on young *in vitro* root tips of untreated (control) plants and of putative polyploids from the colchicine and oryzalin treatments that were segregated using the stomatal characters.

The preliminary evaluation of ploidy levels was carried out using the three youngest but fully developed leaves collected from elongated shoots. These particular leaves were chosen for the assessment as they were initiated after exposure of the explants to the antimitotic chemical treatments. Consequently, the ploidy levels of each sampled leaf and the newly emerging shoot on which it was born were expected to correspond with each other, as they would have been initiated from the same meristem initials (Kermani *et al*., 2003).

In this experiment, the stomatal density from the diploid control plants was used as a baseline to segregate the putative polyploid plants. In all cases, three well-expanded leaf samples were taken from each plant and their stomata on the lower epidermis (abaxial surface) were used for evaluation. To determine stomatal density, a small area (approximately 1 cm^2) on the abaxial side of the leaf was smeared with a thin layer of transparent nail polish and left to dry for 10 min. The nail polish impression was then swiftly removed using a piece of transparent adhesive tape. The tape with the epidermal layer impression was then placed on an object slide and observed under a light microscope (ACCIJ-SCOPE Inc.; Sea

Cliff, NY, USA) at 400× magnification. Hence, the number of stomata and the number of cells were counted and recorded within each field of view (1.0 mm^2) from ten independently sampled leaf areas (van Duren *et al*., 1996). The average stomatal index (SI) of these samples was then calculated and recorded following the formulae stated by Weyers and Meidner (1990):

$$SI = \frac{(\text{Number of stomata per unit leaf area (NSULA)} \times 100)}{(\text{NSULA} + \text{Number of epidermal cells per unit leaf area})}$$

In addition, the stomatal length was measured from each sampled field of view using an ocular-micrometer (400× magnification).

Chromosome counting was carried out on actively growing *in vitro* root tips of untreated (control) plants and of the selected putative polyploids from the colchicine and oryzalin treatments, following the general procedures outlined by Sakhanokho *et al*. (2009). Thereby, 0.5–1.0 cm long tips were collected between 0930 and 1030 hours, washed with tap water and put in 0.002 M 8-hydroxy quinoline and kept at 4 ^oC in a refrigerator for 4–5 h for pretreatment. Pretreated roots were washed again and fixed in Carnoy's solution (3 parts absolute ethanol mixed with 1 part glacial acetic acid) at 4 ^oC for 1 d. Then, the roots were washed and transferred into a 1 N HCl solution and macerated in a hot water bath (60 ^oC) for 5 min prior to their retention in 70% ethanol at 4 ^oC until observation.

For chromosome observation, 1–2 mm of the treated and preserved root tips were cut and chopped into pieces on glass slides. The roots were well squashed on the slide after adding 1–2 drops of 1% aceto-orcein solution and then were covered with a cover slip. The excess aceto-orcein solution was blotted with tissue paper and cells were further squashed gently with the thumb. The slides were then put under a photomicroscope (Labphot-2; Nikon Corp.; Tokyo, Japan) and pictures were taken using 100× magnification with a drop of oil. Finally, the number of chromosomes from the control (diploid) and the putative polyploid plants were counted and compared. The chromosome count of each pre-selected regenerant was repeated at least three times using different root tip samples for confirmation.

The numbers of shoots surviving the chemical treatments were counted and percentages were calculated. Likewise, data on the number of putative polyploids from the stomatal measurements—stomatal number (density) and stomatal length—were collected, and stomatal indices were also calculated using the formula given above.

The experiment was laid out in a completely randomized design with ten replications and three subsamples (nodal explants) per replicate. The data were analyzed using the SAS statistical software (SAS Institute, 2003).

RESULTS AND DISCUSSION

Survival of treated shoots

The survival rate of treated korarima shoots from the lowest concentration of colchicine was lower than that of oryzalin (37% and 67%, respectively). The higher the concentration of the antimitotic chemicals, the higher was the mortality rate (Table 1). Miguel and Leonhardt (2011) reported similar findings indicating the detrimental effects of higher concentrations of these antimitotic chemicals. Based on a preliminary segregation through their general morphology (vigor of the plantlets, color intensity and size of the leaves), the number of putative tetraploid plants from the oryzalin treatment was found to be higher than that from the colchicine treatment (17 and 11 plants, respectively) as shown in Table 2.

Ploidy determination

Stomatal characters

All the putative polyploid shoots from the morphological screening showed distinct differences from their diploid relatives, regarding their stomatal number (density) and length. The stomatal indices were shown to be less sensitive in ploidy discrimination. Accordingly, the highest

number of putative polyploids from the colchicine (5) and oryzalin (9) treatments were obtained from the treatments involving the lowest concentrations of the chemicals used in this study, (125 µM and 10 µM, respectively) as shown in Table 2. The presumed polyploid plantlets generally exhibited reduced stomatal density (3.16–4.20 mm^{-2}) and longer stomata (14.75–16.52 µm) and in some cases lower stomatal indices (5.06–6.48) than the controls that were recorded at a mean of 9.58 mm^{-2}, 11.38 µm and 10.20 for the respective parameters (Table 2, and Figures 1a and 1b). The efficiency of using stomatal characters (the number or size of stomata or both) for preliminary segregation of putative polyploids has also been reported in orchids (Miguel and Leonhardt, 2011), passion fruit (Rêgo *et al*., 2011) and *Salvia hains* (Grouh *et al*., 2011) among others.

In general, korarima plants were observed to be amphistomatous (bearing their stomata on both the abaxial and adaxial surfaces), which is a common character of most herbaceous plant species (Weyers and Meidner, 1990). However, being an obligatory shade-loving plant, the stomata of korarima were found to be relatively abundant on their abaxial surface. In the present study, the overall stomatal characters of the regenerants from the different treatments generally agreed with the preliminary evaluation depicted by the morphological characters. Therefore, those regenerants exhibiting relatively vigorous growth were also observed to possess larger sized stomata but fewer in number (Table 2, and Figures 1a and 1b).

Chromosome count

The chromosomes of korarima were observed to be small in size (Figure 1) and their diploid number was found to be $2n = 2x = 48$. Of the total putative tetraploid korarima shoots selected from the colchicine and oryzalin treatments using plant morphology and stomatal characters (11 and 16, respectively), only three from the 125 µM colchicine (P2) and four from the 10 µM oryzalin (P6) treatments were confirmed to be solid tetraploids through a chromosome count (Table 2, and Figure 2). The diploid and tetraploid chromosome numbers of korarima recorded in this study were also comparable with the diploid and

Table 1 Survival rates (%) of korarima plantlets at 7 d after treatment.

Agent (µM)	Treated	Survived (%)
Control	30	30 (100.00)
Colchicine		
125	30	11 (36.67)
250	30	8 (26.67)
375	30	7 (23.33)
500	30	3 (10.00)
Total	120	29 (24.17)
Oryzalin		
10	30	20 (66.67)
20	30	22 (73.33)
40	30	17 (56.67)
60	30	12 (40.00)
80	30	10 (33.33)
Total	150	81 (54.00)
Grand total	300	140 (51.85)

Table 2 Morphological and cytological changes in korarima after colchicine and oryzalin treatment *in vitro*.

Chemical agent (μM)	Number of shoots survived & tested for polyploidy	Shoot number (% in parentheses) of presumed polyploids from each group based on						
		Morphology	Stomatal characters			Chromosome count		
			No.	LG (mm)	Index	DP	TP	MP
0	30	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	30 (100.00)	0 (0.00)	0 (0.00)
Colchicine								
125	11	5 (45.45)	5 (45.45)	5 (45.45)	2 (18.18)	6 (54.54)	3 (27.27)	2 (18.18)
250	8	2 (25.00)	2 (25.00)	2 (25.00)	2 (25.00)	6 (75.00)	0 (0.00)	2 (25.00)
375	7	2 (28.57)	2 (28.57)	2 (28.57)	2 (28.57)	5 (71.43)	0 (0.00)	2 (28.57)
500	3	2 (66.67)	2 (66.67)	2 (66.67)	0 (0.00)	1 (33.33)	0 (0.00)	2 (66.67)
Total	29	11 (37.93)	11 (37.93)	11 (37.93)	6 (20.69)	18 (62.07)	3 (10.34)	8 (27.59)
Oryzalin								
10	20	9 (45.45)	9 (45.00)	9 (45.00)	1 (5.00)	11 (55.00)	4 (20.0)	5 (25.00)
20	22	7 (31.82)	6 (27.27)	6 (27.27)	1 (4.54)	16 (72.73)	0 (0.00)	6 (100.00)
40	17	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	17 (100.00)	0 (0.00)	0 (0.00)
60	12	1 (8.33)	1 (8.33)	1 (8.33)	0 (0.00)	11 (91.67)	0 (0.00)	1 (8.33)
80	10	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	10 (100.00)	0 (0.00)	0 (0.00)
Total	81	17 (20.99)	16 (19.75)	16 (19.75)	2 (2.47)	65 (80.25)	4 (4.94)	12 (14.81)
Grand total	140	28 (20.00)	27 (19.28)	27 (19.28)	8 (5.71)	113 (80.71)	7 (5.0)	20 (14.29)

Numbers in parentheses represent relative percentages of the plantlets from the total number of shoots tested for polyploidy under their respective category.

Stomatal No. = Mean number of stomata; Stomatal LG = Mean length of stomata; Stomatal index = Relative percentage of the stomatal density over the overall number of stomata and epidermal cells per unit leaf area.

DP = Diploid chromosomes; TP = Tetraploid chromosomes; MP = Mixoploid chromosomes.

tetraploid chromosome numbers of cardamom (*Elettaria cardamomum*) (Sudharshan, 1989), which is a very close relative of the former.

The relative efficacy of colchicine and oryzalin has also been compared in different plant species, with similar results to the present finding of a higher chromosome doubling efficiency at relatively lower concentrations of oryzalin rather than using colchicine being reported in various plant species, including *Lilium* spp. (van Tuyl *et al*., 1992), *Miscanthus sinensis* (Petersen *et al*., 2003), *Rosa* spp. (Kermani *et al*., 2003), and passion fruit (Rêgo *et al*., 2011) among others. In general, this could be ascribed to the inherent higher affinity of plant tubulins to oryzalin than to colchicine (Morejohn *et al*., 1987).

CONCLUSION

The present work can be considered as pioneering the determination of the diploid chromosome number of korarima, which was found to be 48. *In vitro* induction of tetraploidy in korarima was attained through the treatment of cultured shoots with a solution of 125 µM colchicine or 10 µM oryzalin, which were the lowest concentrations tested. In general, the relatively less toxic nature of oryzalin together with its affordability makes it by far the best candidate for future *in vitro* polyploid induction work with korarima.

In all cases, tetraploid korarima plantlets revealed vigorous growth, possessing thicker and

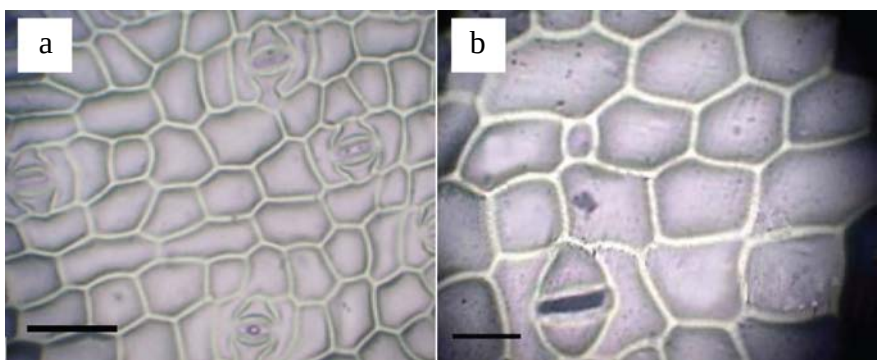

Figure 1 Stomata from the abaxial leaf epidermis: (a) Diploid and (b) Tetraploid korarima shoots. (Scale bar = 25µm.)

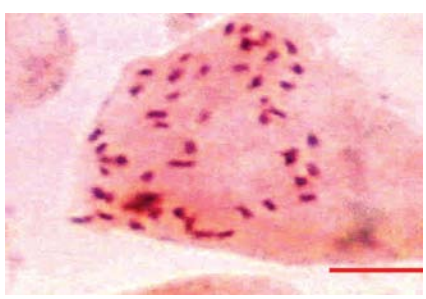

Figure 2 Mitotic chromosomes of a diploid korarima plant (2n = 48). (Scale bar = 10 µm.)

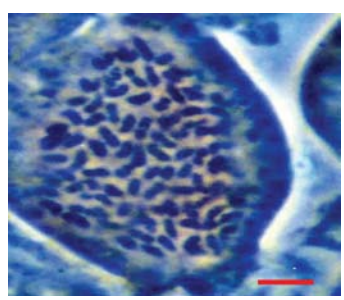

Figure 3 Mitotic chromosomes of a tetraploid korarima plant (2n = 4x = 96). (Scale bar = 10 µm.)

large-sized leaves that were deep green in color. Of the different morphological characters evaluated, those related with stomata (density and length) could serve as a quick technique to segregate and pre-select putative polyploid plants. However, these preliminary results from morphological observations must be confirmed using other more reliable techniques such as chromosome count or flow cytometry.

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