RESEARCH ARTICLE

# Ploidy levels in Citrus clementine affects leaf morphology, stomatal density and water content

**Diego Padoan[1]*, Amr Mossad[1,2], Benedetta Chiancone[1], Maria Antonietta Germana[1], Patan Shaik Sha Valli Khan[1,3]**

[1]Departemento DEMETRA, Facolta di Agararia, Unieversita degli Studi di Palermo, Palermo, Italy.
[2]Agricultural Engineering Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.
[3]Department of Botany, Yogi Vemana University, Vemanapuram, Andhra Pradesh, India.

*Corresponding author: pssvkhan@yahoo.com
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**ABSTRACT:** The objective of the present study was to understand the relationship among leaf morphology, stomatal characteristics and water relations in triploids generated through anther culture and their counterpart diploid plant of *C. clementina*. Triploid plants possessed small and narrow leaves as compared to diploid plant as evident by less leaf length, leaf width and leaf area. By contrast, the leaf index was observed to be more in triploids than haploid ones. Flow cytometric analysis re-confirmed the ploidy levels of heterozygous plant Hd as diploid and the ploidy of Th1, Th2, Th3 and Th4 plants as triploids. A positive relation was found between ploidy level and stomatal guard cell length and width, whereas a negative relation was observed between the stomata density and ploidy level. The stomatal density was reported to be 6.2±0.2 stomata per $\mu m^2$ in diploid plant, while stomatal density varied between 3.0 and 3.6 stomata per $\mu m^2$ in triploids. Leaf relative water content (RWC) was slightly higher in triploids (90.8 to 93.1%) than diploid (89.5%). The leaf water loss was found to be marginally higher in diploid than in triploid plants. Our results show that increase in ploidy level from diploids to triploids caused an effect on leaf morphology and stomatal characteristics with probable consequences to water relations of leaves. This research will serve as an important basis for future work on complete analysis of both morphological and behavioural traits of the leaf stomata and transpiration rates in relation to diploid *versus* triploid plants.

**KEYWORDS:** anther culture, citrus, diploid, relative water content, stomata, triploid, water loss.

## INTRODUCTION

Citrus is one of the most predominant fruit crops grown globally, with great nutritional, medicinal and economic importance. It is among the most challenging plants to improve through conventional breeding methods due to their complicated reproductive biology that frequently inhibits sexual hybridization as well as difficulty in segregation of zygotic populations for selection (Grosser et al. 2000). The creation of triploid hybrids is an important breeding strategy to develop new seedless citrus commercial varieties. Citrus triploid hybrids have been produced by crossing tetraploid female parents (4x) with diploid pollen parents (2x) or diploid seed parents (2x) with tetraploid parents (4x). Triploid hybrids can also be recovered from the union of a 2n megagametophyte with haploid pollen (Esen and Soost 1971). The frequency of such crossings is generally low and requires effective methodologies for embryo rescue and ploidy evaluation (Ollitrault et al. 2008). Alternatively, *in vitro* regeneration of plants from endosperm offers one-step approach for triploid production (Thomas and Chaturvedi 2008).

Clementine is a natural hybrid between sweet orange and common mandarin selected in 1902 in Algeria. All the cultivars of Clementine have ascended from the initial "Fina" Clementine by the build-up of spontaneous mutations. Among them, "Nules" Clementine, a direct mutation of "Fina", is the most important commercial cultivar extensively cultivated in the Mediterranean basin for fresh-fruit market. Moreover, it has been an essential germplasm for mandarin breeding and designated as a target to obtain the haploid genotype for whole genome sequencing (Aleza et al. 2009). Anther culture has been already engaged for the production of androgenic haploids

to speed and support breeding programs in *Citrus clementina* Hort.ex. Tan., with homozygous lines with different ploidy levels such as haploids, aneuploids, doubled haploids and triploids lines achieved through anther culture being used. The triploid plants derived from anther culture were grown under greenhouse, showing vigorous growth and considerable leaf morphological variation as compared to diploid ones (Germanà et al. 2005). Sunderland et al. (1974) demonstrated the origin of triploids and tetraploids from anther culture. Single nucleus of an embryogenic grain divides asymmetrically into a generative and a vegetative nucleus. An endoreduplicated generative nucleus fuses with a vegetative nucleus to form a triploid embryo, whereas the fusion of an endo-reduplicated generative nucleus and two vegetative daughter nuclei leads to formation of a tetraploid embryo. The processes of androgenesis in plants have been more recently reviewed by Segu-Simarro and Nuez (2008) and Germanà et al. (2011a, 2011b).

In the genus *Citrus* and other members of the sub-family Aurantioideae, the basic chromosome number is x=9. Practically, all wild and cultivated forms of *Citrus* are diploid and comprise small sized chromosomes (Krug 1943). Hence, ploidy level analysis in *Citrus* by cytogenetic methods is a slow and inadequate process when large populations of plants have to be analysed. However, detecting polyploid seems to be convenient with molecular markers and the use of flow cytometry is an easier and much faster method, which was also adopted for citrus (Germanà et al. 2005, Aleza et al. 2010).

Stomata are specialized epidermal structures that act as turgor-operated valves for gas exchange. They are formed by two kidney-shaped guard cells in dicots that encircle a pore and links intercellular spaces inside the leaf to the atmosphere. This continuity is critical for plant survival because it allows carbon dioxide to reach mesophyll chloroplasts for photosynthetic reactions. Regulation of pore width restricts water loss, being controlled by environmental and plant parameters through complex signal transduction pathways. Stomatal frequency, guard cell length and stomatal plastid number have often been used as morphological marker for identifying ploidy levels in many plant species (Mishra 1992, Beck et al. 2003, Yuan et al. 2009, Ye et al. 2010). In general, stomata and epidermal cell frequency per unit leaf area diminished while stomata guard cell length improved with an increase in ploidy (Yuan et al. 2009, Ye et al. 2010). In the genus *Citrus*, stomatal frequency and size were previously studied in several species viz. *Citrus paradisi* Macfed var. Marsh, *C. limon* (L.) Burm. f. Var. Monachello L CNR13, *C. sinensis* (L.) Osbeck var. Valencia, AA. CNR 23 and AA CNR30, *C. aurantium* L. and *C. reticulata* var. Mandarino Tradivo Claculli CNR 19 (Germanà et al. 2002) and Kinnow mandarin ( Jaskani et al. 2002). In general, leaves of *in vitro* grown plants exhibited higher stomatal frequencies than those of *in vivo* plants. The correlation between high stomatal density and extent of water loss had been well studied in tissue culture derived plants. The major reason for high mortality rate during *ex vitro* transfer relies on excessive water loss due to high stomatal densities (Sha Valli Khan et al. 2009).

The relation between stomatal characteristics and water relations at dissimilar ploidy levels in this species has received little attention. Therefore, this study was conducted to describe leaf morphology in different Citrus clementina ploidy levels, looking specifically for evidence of stomatal density and water loss changes thereof.

## MATERIAL AND METHODS

**Plant Material and Leaf Morphology:** Shoot apices (2 to 3 mm in length) of anther derived triploids (Th1, Th2, Th3 and Th4) and diploid (Hd) were selected and grafted *in vitro* onto 20 d old etiolated seedlings of *C. clementina*. After effective hardening under greenhouse, micrografts were transplanted to polythene bags filled with sand, peat moss and soil (1:1:1) and kept under natural environmental conditions. The plants were irrigated every other day to the maximum holding capacity of substrate throughout the experimental period and were grown under maximum photosynthetic photon flux density (PPFD) of 1,000 μmol $m^{-2}$ $s^{-1}$, air temperature varied between 25 and 30°C and air relative humidity was 50±20%.

Triploid plants showed variation with regard to important morphological traits such as the level of leaf morphology and thorniness as compared to diploid plant. Thorniness, vigorous growth, alternate bearing in early years, physical differences in fruit characters and slowness to flower and to bear fruit are described as juvenile traits in Citrus (Cameroon and Frost, 1968). Therefore, 15 mature and fully expanded leaves from actively growing shoots of diploid (Hd) and triploid (Th1, Th2, Th3, Th4) plants were sampled to determine leaf area, width and length with leaf area meter usage (Delta-T devices, Cambridge, United Kingdom) to study the variation in leaf morphology.

**Ploidy Evaluation:** Ploidy level of Th1, Th2, Th3, Th4 and Hd lines was determined by flow cytometry. Leaf samples (50 $mm^2$) were chopped with razor blade in presence of 1 mL nuclei extraction buffer (Cystain UV Precise P, Partec GmbH, Münster, Germany) in a Petri plate. The samples were filtered through a 30 μm nylon gauze Partec Cell Trics directly into a sample tube and stained with 1 mL of 4',6-diamidino-2-phenylindole stain (DAPI, Partec). Following 5 min incubation at room temperature, stained

samples were run in a ploidy analyser of flow cytometer (Cell analyser PA II) equipped with a HBO high pressure mercury lamp. Histograms were analysed using the Partec FloMax v2.0, which determines peak position, coefficient of variation (CV) and the relative ploidy index of samples.

**Stomatal Characteristics:** Stomatal distribution was studied from epidermal impressions of the adaxial and abaxial surfaces of mature, fully expanded leaves (Sampson 1961). Epidermal impressions were made using methyl acetate UHU extra adhesive (UHU GmbH & Co. KG, Germany) smeared uniformly to the middle portion on either side of the midrib and away from the leaf margins. This leaf position has guard cell dimensions and stomatal density that represent the average of the entire leaf (Beaulieu et al. 2008). After 5 min, the dried membrane was cautiously skinned off and mounted on microscope slide. Size and counts measurements were taken using an AXIOPHOT Fluorescence microscope (Zeiss, GmbH, Germany). The guard cell length and width were measured separately instead of total guard cell area because of the dynamics of stomatal movement. When stomata open or close the short axis (ventral and dorsal lengths) of the guard cells, the long axis remains the same (Willmer and Fricker 1996). Guard cell lengths (µm) were measured to the nearest micrometer viewed at 40x magnification. Stomatal density was assessed by counting the number of stomata per field (200x200 µm) of view at 40x magnification. These values were then converted to stomata per $\mu m^2$. Average minimum sample size was three leaves per plant and three fields per leaf to determine stomata density. Nine measurements were taken for guard cell length and width.

**Relative Water Content:** The leaf water status in Th1, Th2, Th3, Th4 and Hd plants was estimated through the relative water content (RWC), according to Weatherley (1950). The fully expanded leaves were taken at 9:00 am and immediately weighed (fresh weight – FW). In order to obtain the turgid weight (TW), the leaves were placed in distilled water inside a closed 50 mL Falcon tubes and kept in refrigerator at 4°C for at least 48 h to minimise respiration losses. Afterwards, the leaves were placed in a preheated oven at 70°C for 48 h to obtain the dry weight (DW). Values of FW, TW and DW were applied to calculate RWC, using the following equation: RWC (%)=[(FW-DW)/(TW-DW)]x100.

**Leaf Water Loss:** Fully expanded, uniform and mature leaves of Th1, Th2, Th3, Th4 and Hd plants were excised between 10:30 am and 11:30 am for determining the leaf water loss (WL). The moisture (if any) on the surface of leaves was gently removed with a filter paper and, immediately, their fresh weight ($FW_0$) was recorded. The leaves were permitted to transpire by keeping their abaxial side up on clean bench at room temperature of 25 to 28°C and air relative humidity of 65%. Each leaf was weighed at 30 min ($FW_t$) interval thereafter for 24 h. Finally, all the leaves were oven-dried at 70°C for 48 h and re-weighed to obtain DW. Values of $FW_0$, $FW_t$ and DW were applied to calculate WL, using the following equation: $WL\ (\%)=[(FW_0\text{-}DW)\text{-}(FM_t\text{-}DW)/(FW_0\text{-}DM)]x100$.

**Statistical Analysis:** Fifteen fully expanded leaves were taken and the experiments were repeated once. The data were subjected to statistical analysis using standard deviations of the mean, and thereafter to analysis of variance. Mean comparisons were carried out using Duncan's Multiple Range Test (a=0.05).

## RESULTS

**Leaf Morphology:** Triploids exhibited shorter internodes, presence of more thorns, robust appearance and vigorous growth than heterozygous diploid plant. Visual observations were also made with regard to leaf morphology, particularly leaf shape and colour of upper and lower surfaces (Figure 1). A comparison of the leaf length, width, area and index was made between triploid and diploid plants (Table 1). Leaf length, width and area were higher in diploids as compared to triploids. The leaf index (the ratio of leaf length to leaf width) was higher in Th1 (3.3), followed by Th2 (3.1), Th3 and Th4 (3.0) and diploids (2.8). The difference in leaf index indicated the presence of slightly smaller and narrower leaves in triploid than in diploid plants. Most of triploid leaves rolled upwards at the edges, presenting a spoon like leaf shape. This phenomenon was not observed in diploid leaves.

**Determination of Ploidy:** Flow cytometric analysis based on peak position, CV and the relative ploidy index confirmed that the Hd plant had the normal diploid chromosome number (Figure 2, Peak number 1; Figure 2, Peak number 2).

**Stomatal Characteristics:** The adaxial leaf surface is dark green and without stomata in Hd, Th1, Th2, Th3, and Th4 plants. Microscopic observations of leaf surface imprints confirmed the presence of stomata only on the abaxial leaf surface of triploid and diploid plants. Stomata were marginally sunken into the leaf epidermis and the shape was oval regardless of ploidy level. However, size and density of stomata varied according to ploidy level, with triploids showing longer and wider stomata as compared to diploid plants (Table 2, Figure 3). A negative

association was observed with regard to the stomatal density *versus* ploidy level, with stomatal density being higher in diploid plants than in triploid ones. The stomatal density per leaf surface was reported to be 6-7 stomata per $\mu m^2$ in diploid plant, while stomatal density was reduced to 3-4 stomata per $\mu m^2$ in triploids (Th1, Th2, Th3 and Th4).

**Relative Water Content and Water Loss:** Leaf RWC was found to be 92.17% in Hd (diploid), followed by 90.8% in Th1, 89.5% in Th2, 93.1% in Th3 and 91.5% in Th4. The water loss from leaves of diploid plant was considered marginally higher than triploids. This was observed at each 30, 60, 90 and 120 min interval of air-drying. Leaves from diploid plant had an average

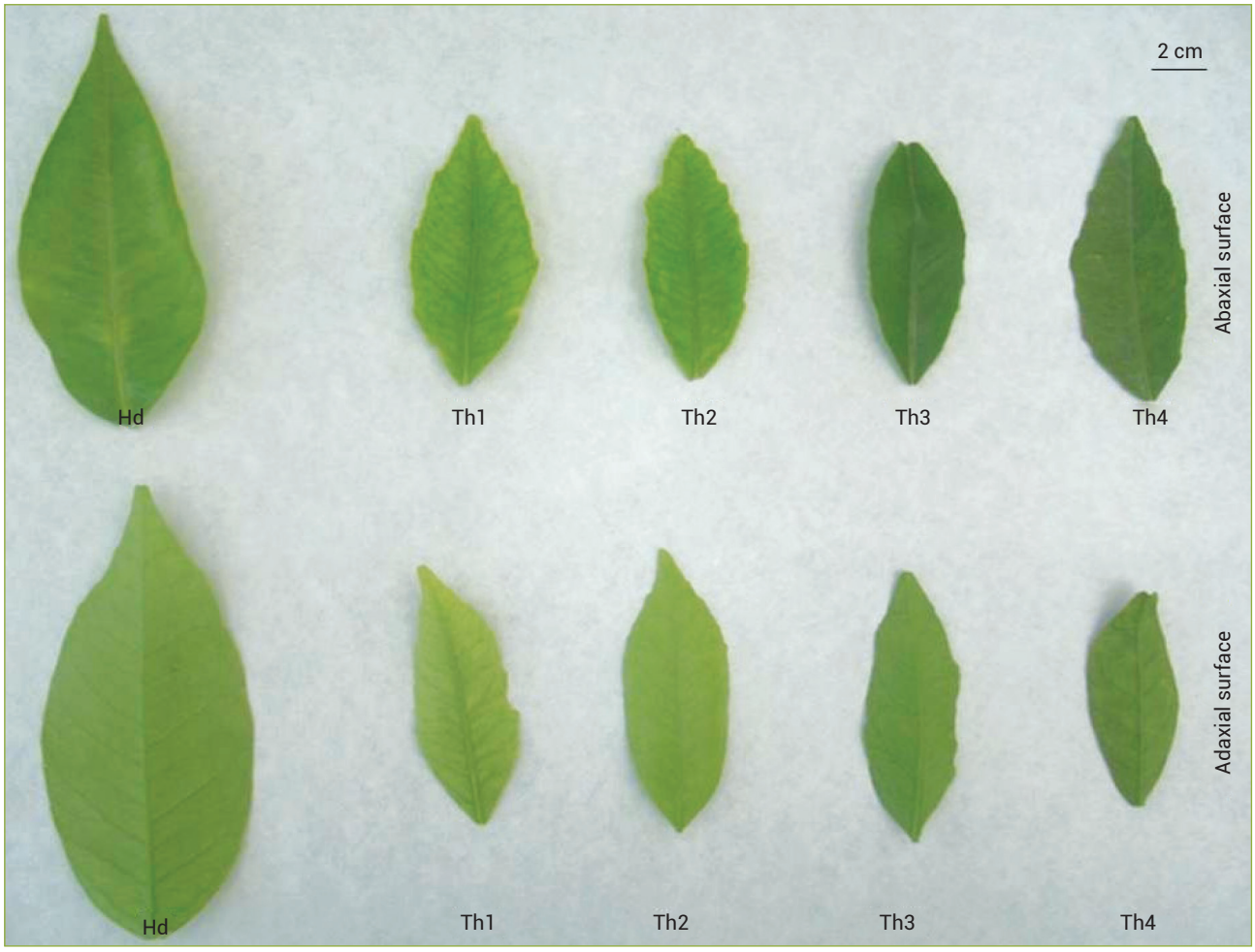


**Figure 1.** Leaf abaxial and adaxial surfaces of of diploid (Hd) and triploids (Th1, Th2, Th3 and Th4) of *C. clementina* (Scale).

**Table 1.** Leaf morphology of diploid (Hd) and triploids of *C. clementina*

| Plant | Leaf morphology | | | |
|---|---|---|---|---|
| | Leaf area | Leaf length (mm) | Leaf width (mm) | Leaf index |
| Hd | 28.5±9.5$^a$ | 11.06±1.8$^a$ | 4.1.0±0.8$^a$ | 2.8±0.3$^a$ |
| Th1 | 7.5±1.4$^b$ | 62.0±0.6$^b$ | 18.0±0.2$^b$ | 3.3±0.5$^b$ |
| Th2 | 5.9±0.9$^b$ | 53.0±0.5$^b$ | 17.0±0.2$^b$ | 3.1±0.5$^b$ |
| Th3 | 8.3±1.1$^b$ | 63.0±0.6$^b$ | 21.0±0.2$^b$ | 3.0±0.4$^b$ |
| Th4 | 6.6±1.3$^b$ | 56.0±1.4$^b$ | 18.0±0.2$^b$ | 3.0±0.7$^b$ |

The mean of 15 leaves±standard deviation; a, b, c letters indicate significance of difference between means at the $\alpha$=0.05 level. There was statistically significant difference in the five groups (confidence level of 95%) for leaf dimensions.

water loss of 10 and 26% after 30 and 120 min, respectively. In contrast, the water loss from leaves of triploids such as Th1, Th2, Th3 and Th4 ranged between 5-10 and 17-24% after 30 and 120 min, respectively (Figure 4).

## DISCUSSION

The anther derived homozygous triploid plants (Th1, Th2, Th3 and Th4) of *C. clementina* displayed vigorous vegetative growth and morphological variation of leaves as compared to heterozygous diploid (Hd) plant. In contrast, haploid plants of Clementine, obtained either by androgenesis or gynogenesis, displayed a weak appearance and poor growth and this is attributed to the expression of recessive deleterious or lethal genes (Aleza et al. 2009). Triploid plants possessed small and narrow leaves as compared to diploid plants as evident by less leaf length, leaf width and leaf area. By contrast, the leaf index is higher in triploids than in diploid. The leaf index is defined as the ratio of leaf length to leaf width. It is a key parameter, in particular, in studies of leaf shape that focus on plasticity, natural variation and environmental adaptation (Tsukaya 2002). The leaf index of several plants reveals plasticity in response to environmental or physiological conditions. Changes in leaf index can be ascribed to two different histologic events such as variations in cell proliferation as well as in cell expansion in leaf blades (Tsukaya 2005). It has been repeatedly reported that the polyploidization also increases the sizes and area of leaves (Ye et al. 2010). Furthermore, the leaves of triploids rolled upwards at the edges, presenting a spoon like leaf shape. The unbalanced growth between the two different tissues in the same blade might have resulted in uneven growth of leaf blade and leaf rolling, as

**Table 2.** Stomatal characteristics at different ploidy levels in *C. clementina*

| Plant | Stomatal density ($mm^2$) | Guard cell length (µm) | Guard cell width (µm) |
|---|---|---|---|
| Hd | 6.2±0.2 [b] | 19.3±1.8 [a] | 15.03±1.5 [a] |
| Th1 | 3.2±0.6 [a] | 21.9±1.4 [b] | 17.34±1.1 [b] |
| Th2 | 3.0±0.3 [a] | 21.5±1.3 [b] | 17.35±2.0 [b] |
| Th3 | 3.3±0.5 [a] | 22.4±2.6 [c] | 18.23±2.1 [c] |
| Th4 | 3.6±0.8 [a] | 22.2±1.7 [c] | 17.42±1.3 [b] |

The mean of 15 leaves±standard deviation; a, b, c letters indicate significance of difference between means at the $\alpha$=0.05 level. ANOVA analysis showed that there is a (statistically) significant difference among the mean of the stomata density at significance level of 5%.

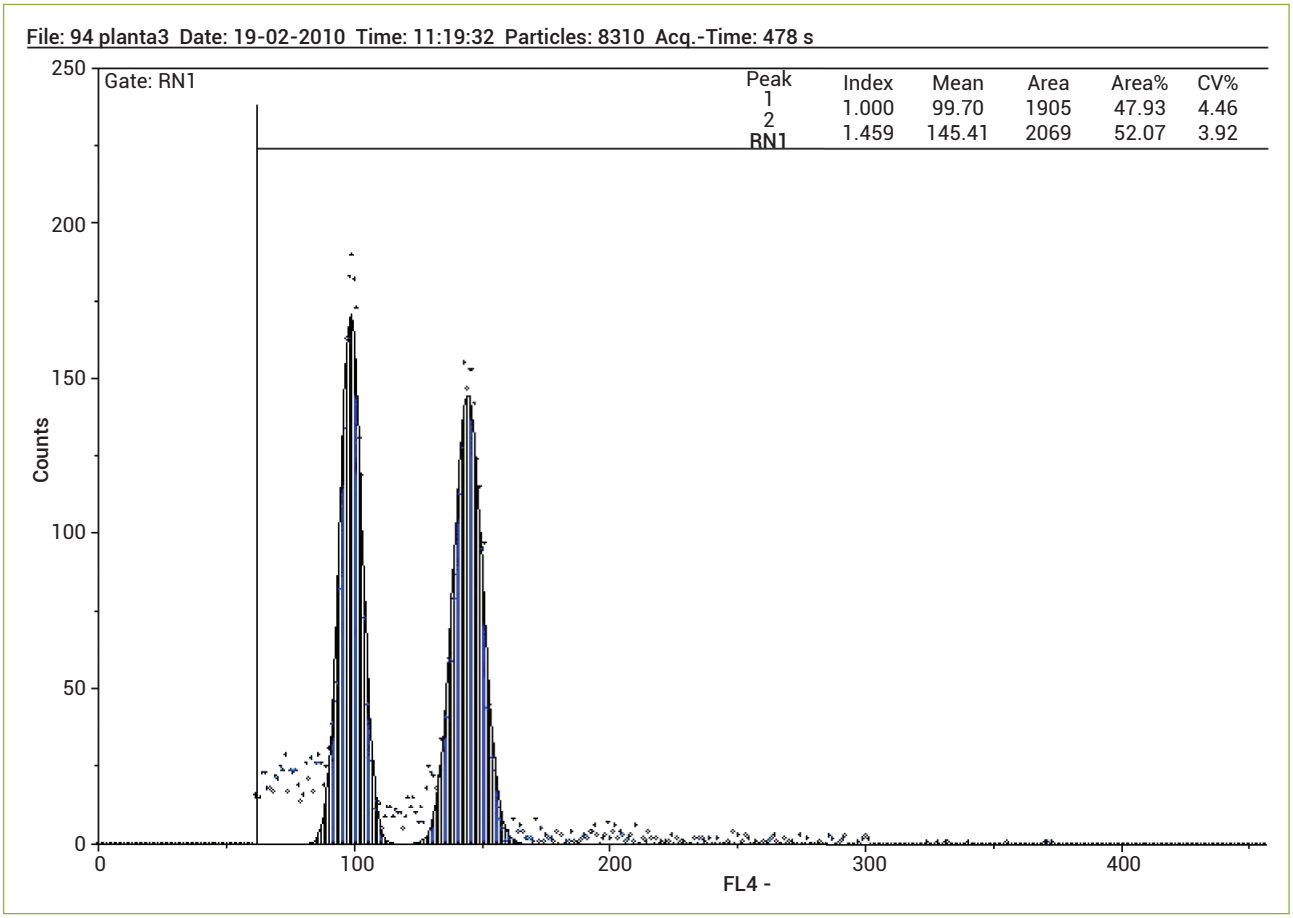


**Figure 2.** Example of flowcytometric histogram of relative DAPI fluorescence (Peak 1: diploid; peak 2: triploid).

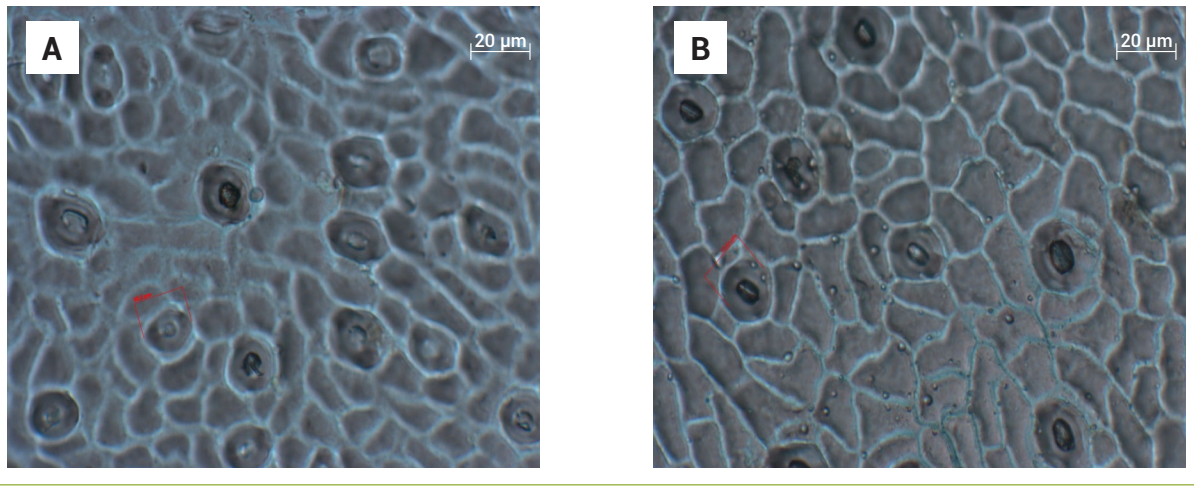


**Figure 3.** (A) Stomatal density in leaves of diploid (scale bar=20 µm); (B) stomatal density in leaves of triploid of *C. clementina* (scale bar=20 µm).

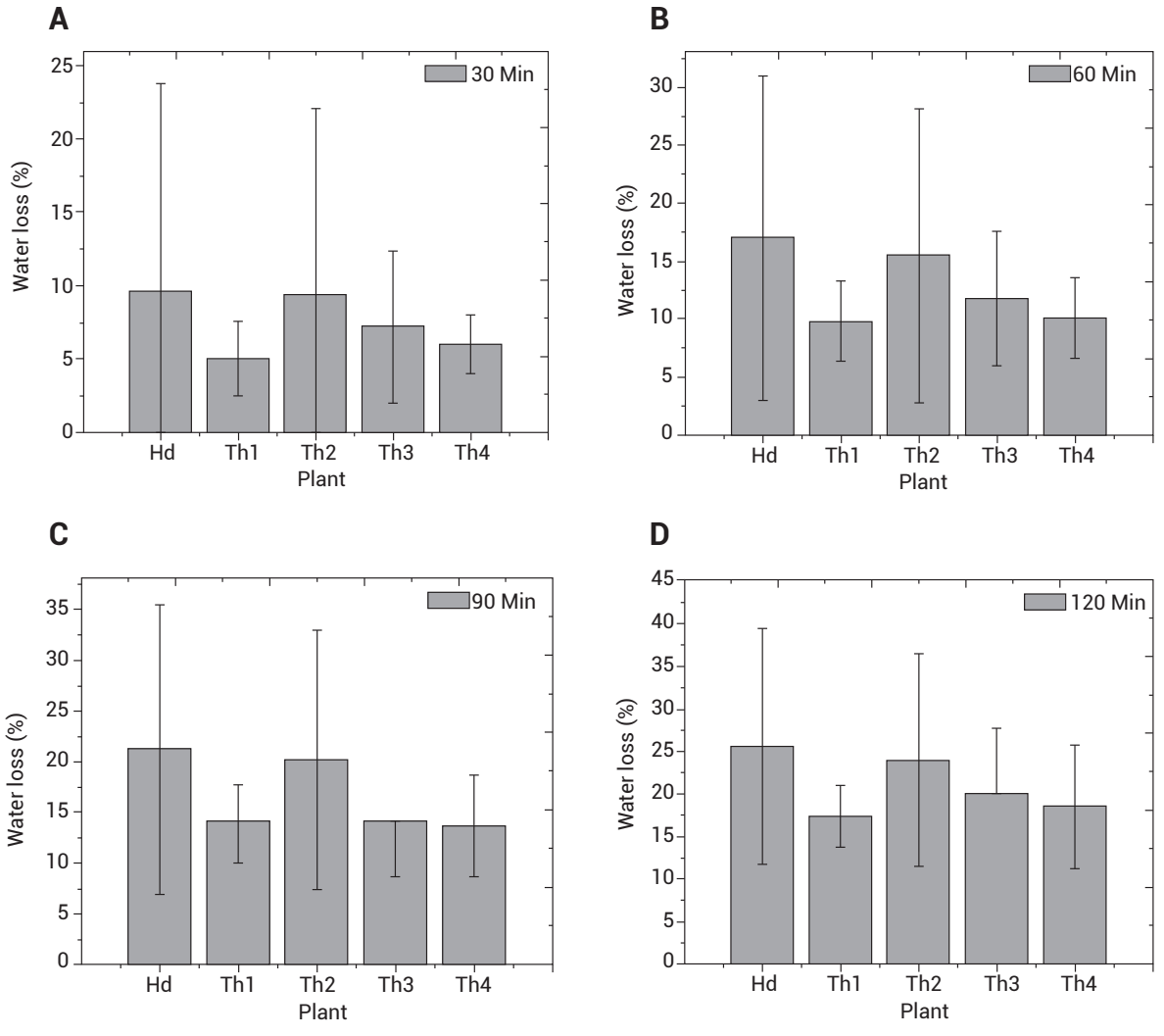


**Figure 4.** Water loss from leaves of diploid (Hd) and triploids (Th1-Th4) of *C. clementina* at 30, 60, 90, 120 min intervals, respectively.

previously reported by Zhou et al. (2002). Leaf rolling reported to effectively impact water and energy balance in plants by altering the representative leaf dimension and the conductance of water, heat and exchange of gases particularly carbon dioxide (Redmann 1985). Andersen et al. (1990) carried out ploidy assessments in carrot plants obtained from anther cultures over a period of three years. Each year, diploid plants outnumbered haploids and tetraploids. In the first year, 60% of the plants were diploids, 82% in the second one, and 76% in the third year. In the experiment presented here, all four triploids tested were stable and there is no change or reversion in their ploidy levels since 1994. These results therefore suggested that the anther culture technique can be used to obtain stable triploids.

The diploid (Hd) plant was associated with smaller guard cells as compared to triploids (Th1, Th2, Th3 and Th4). The stomatal frequency (number per unit leaf surface) found to be 6-7 stomata per $\mu m^2$ in diploid and 3-4 stomata per $\mu m^2$ of leaf surface in triploids. The low stomatal frequency is in accordance with the findings of Jaskani et al. (2002), who reported 7-8 stomata per unit leaf area in diploids and 3-4 stomata per leaf area in tetraploids (3-4) of Kinnow mandarin. The coordination of the size and frequency of stomata is an important trait for maximizing water use efficiency across environments. The expansion of epidermal cells or guard cells influences stomatal density through compensatory mechanisms associated with cell size and cell number (Beaulieu et al. 2008). The results suggest that increase of ploidy level from diploidy to triploidy enhanced guard cell length and reduced stomatal density. This result is consistent with the observations made by Beck et al. (2003), who found a positive relationship between ploidy level and stomatal length and an inverse relationship between the frequency of stomata and ploidy level in diploid and tetraploids of *Acacia mearnsii* (de Wild). Ploidy levels of plants can be determined using direct methods, i.e. chromosome counting, or indirect methods, i.e. flow cytometry, stomatal size and frequency, chloroplast number of the guard cells and morphological observations. In addition to flow cytometry, the present study also demonstrated usefulness of measuring stomatal guard cell length and stomatal density in plants with two different ploidy levels. This method may be considered as an easier, faster, more economical and alternative for the determination of ploidy levels in *C. clementina*. This result was consistent with previous studies reported for Kinnow mandarin (Jaskani et al. 2002), *Acacia mearnsii* (Beck et al. 2003) and *Brassica oleracea* (Yuan et al. 2009).

In dry environments, small stomata allow a rapid response to water stress, while high densities allow maximization of $CO_2$ diffusion during optimal photosynthetic conditions (Aasamaa et al. 2001). Increased stomatal density is associated with greater stomatal conductance and transpiration rates, which are thought to be necessary for moving water and nutrients through longer xylem pathways. In addition, small stomata allow greater stomatal resistance and stomatal control during water stress conditions (Hetherington and Woodward 2003). Therefore, decrease in stomatal density in triploids may be one of the causes for less percentage of water loss from leaves of triploids in comparison with the leaves of diploid plant. Leaf RWC was varied between 89.5 to 93.1% in triploids and diploid. Difference in leaf RWC between triploid and diploid plants was statistically not significant. Leaf RWC is a valid index of the water equilibrium in plants (Gong et al. 2006). It is used to study the extent of dehydration and assess the cellular damage and severity of drought in plants (Flexas and Medrano 2002). Leaf RWC did not show significant correlation with two different levels of ploidy tested in the present study.

In conclusion, we report for the first time about the changes in leaf morphology, stomatal traits and water relations in *C. clementina* with diploid and triploid levels. Our results showed that increase in ploidy level from diploid to triploids (1) caused variation in leaf morphology particularly leaf length, leaf width, leaf area and leaf index, and (2) decreased stomatal density and resulted in an increase in length of stomatal guard cell. In addition, that alteration of leaf morphology and decrease in stomatal density considerably reduced water loss from the leaves of triploids as compared to diploids.

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