CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

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In vitro induction of polyploidy in *Tacca leontopetaloides*

Master's thesis

Prague 2018

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Declaration

25th April, 2018

I Samuel Agyei declare that I have worked on my diploma thesis titled "*In vitro* induction of polyploidy in *Tacca leontopetaloides*" by myself and I have used only the sources mentioned at the end of the thesis. As the author of the diploma thesis, I declare that the thesis does not break copyrights of any third person.

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Samuel Agyei

Acknowledgement

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along to the completion of my diploma thesis. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I would first like to thank my thesis supervisor Ing. Iva Viehmannová, Ph.D. of the Faculty Tropical AgriSciences at Czech University of Life Sciences, Prague. The door to Dr. Viehmannová office was always open whenever I ran into a trouble spot or had a question about my research or writing. She consistently allowed this paper to be my own work but steered me in the right the direction whenever he thought I needed it.

I would also like to thank my thesis co-supervisor Dr. Aaron Tetteh Asare of the College of Agriculture and Natural Sciences at the University of Cape Coast, Ghana. Without his passionate participation and input, my research work in Ghana could not have been successfully conducted.

I would also like to acknowledge Mr. Solomon Mensah for his support and assistance during my research work at the Plant Tissue Culture laboratory at the University of Cape Coast.

I must express my very profound gratitude to my parents and to my entire family for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

I am grateful to the Czech Republic Government for financing my study and stay in the Czech Republic through the Czech Republic Government Scholarships for Developing Countries.

Finally, this research was financially supported by the Internal Grant Agency of Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague IGA (Project Nos. 20165014 and 2017501).

Abstract

Tacca leontopetaloides is a tropical tuberous food crop which contains starch of a raw material source potential for industries. The aim of this study was to induce *in vitro* polyploidization in *T. leontopetaloides* using oryzalin as an antimicrotubule agent, to detect polyploids using flow cytometry and to assess polyploids *in vitro* and in field conditions of Ghana. Shoots were treated *in vitro* with oryzalin (25 µM and 30 µM) for 24 or 48 hours. The highest polyploidization efficiency of 10% was achieved with 25 µM oryzalin regardless of time duration and with 30 µM for 24 hours exposure time. In total, seven of 80 (8.75%) tetraploids were obtained from all treatments. The highest survival rate of shoots (90%) was achieved with 25 µM for 24 hours exposure time. Morphology of the tetraploid plants was evaluated on MS medium without plant growth regulators (PGRs). Tetraploid plants showed altered morphological characters such as increased leaf width/length ratio, rounder leaf shape and dark green colouration as well as significantly higher number of roots (4.85 ± 0.66 roots/explant) and root length (0.91 ± 0.10 cm) compared to diploids with lower number of roots (2.95 ± 0.52 roots/explant) and root length (0.62 ± 0.10 cm) respectively. No significant difference in plant height was found between tetraploid and the diploid plants. Cultivation of tetraploid and diploid plants on multiplication MS salts medium fortified with 0.05 mg l^{-1} NAA and 0.1 mg l^{-1} zeatin showed increased in the number of shoots (5.06 ± 0.19 shoots/explants) in diploids compared to tetraploid plants (4.43 ± 0.16 shoots/plant). Tetraploid and diploid plants were evaluated *ex vitro* under tropical conditions of Ghana. The survival rate after six weeks of acclimatization in the greenhouse was 100% and 91.6% for diploid and tetraploid respectively. After twelve weeks under field conditions, however, the survival rate was 60% and 80% for diploid and tetraploid respectively showing better adaptation of tetraploid plants to field conditions. Tetraploid plants produced a higher number of tubers per plants as well as increased in tuber size. The tetraploid plants showed slower growth compared to diploids. These results proved that *in vitro* treatment of *T. leontopetaloides* shoots with oryzalin solution is an effective procedure for chromosome doubling and tetraploid plants may be used as a material with modified morphology, growth and yield characteristics for further breeding and the generation of novel varieties of *T. leontopetaloides*.

Keywords: Flow cytometry, *in vitro* culture, oryzalin, ploidy level, *Tacca leontopetaloides,* morphological characteristics

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List of the abbreviations

BAP	6-benzylaminopurine
CULS	Czech University of Life Sciences
DNA	Deoxyribonucleic acid
DAPI	4´,6-diamidino-2-phenylindole
DMSO	Dimethylsulfoxide
MS	Murashige and Skoog medium (1962)
NAA	α-naphthaleneacetic acid
PGR	Plant growth regulator
PROSEA	Plant Resources of South-East Asia
rDNA	Ribosomal deoxyribonucleic acid
ZEA	Zeatin

1. Introduction and literature review

1.1. Introduction

Polynesian arrowroot (*Tacca leontopetaloides* (L.) Kuntze) is a perennial species of flowering plant which belongs to the family Dioscoreaceae. This species is native to tropical Africa, Southeast Asia, and Northern Australia and it is known to have been domesticated in the Pacific Islands through early human migration due to its importance as a food source (Ukpabi et al. 2009; Ubwa et al. 2011). When staple foods such as Yam and cassava become scarce in Nigeria, the tubers of *T. leontopetaloides* are considered as an alternative food source (Kay 1987). This species is also used as a medicinal plant. In India, the plants have been over-collected for its medicinal properties resulting in a local depletion (Bagul & Yadav 2007). The starch from the tubers has attracted research attention in recent years as a potential raw material for biodegradable plastics (Makhtar et al. 2013) and tablets formulation in the pharmaceutical industry since it possessed similar physiochemical properties to maize starch (Kunle et al. 2003).

T. leontopetaloides can be propagated by seeds. However, due to poor seed germination, vegetative propagation by the use of tubers is mostly practiced by farmers (Spennemann 1994; Borokini et al. 2011). *T. leontopetaloides* possess self-pollinating, hermaphroditic and perfect flowers like other members of the *Tacca* genus (Borokini et al. 2012) resulting in low genetic variability. Biotechnology, therefore, present the opportunity for the development of new genotypes of this species.

Polyploidy is defined as the possession of three or more complete copies of the nuclear chromosome set. Polyploidy in plants is believed to have been discovered a century ago and is considered to be an important feature of chromosome evolution (Ramsey & Schemske 1998). *In vitro* induction of polyploidy has become a common biotechnological method for the improvement of crop yield and chemical composition. In agricultural and horticultural practices, polyploidy brings about gigantism in all or some characters. Induction of polyploidy in *T. leontopetaloides* could be useful for the improvement of the yield and the chemical composition of its tubers.

Although this species remains a wild and under-utilized crop, it has the potential for diversification away from over-reliance on staples crops for food and industrial raw materials.

Therefore this study was carried out to induce *in vitro* polyploidization in *T. leontopetaloides* using oryzalin as an antimicrotubule agent, to detect polyploids using flow cytometry and to assess polyploids *in vitro* and in field conditions of Ghana.

1.2. Literature review

1.2.1. Taxonomical classification of *Tacca leontopetaloides*

Tacca leontopetaloides (L.) Kuntze belongs to the kingdom Plantae, the order Dioscoreales, and family Dioscoreaceae. *Tacca* genus was treated in its own family Taccaceae but Caddick et al. (2002) after doing an extensive study of the order using analysis of three genes, rbcL, atpB and 18S rDNA, and morphology to examine relationships of nearly all genera of the order Dioscoreales expanded Dioscoreaceae family to incorporate the *Tacca* genus and this was accepted by Angiosperm Phylogeny Group (APG II 2003).

1.2.2. Reproductive biology of *Tacca leontopetaloides*

Tacca leontopetaloides is characterized by inconspicuous dark-green inflorescences with small bracts and short bracteoles. Floral traps, the absence of nectar, and a decaying odour are common features of the sapromyiopholous. Drenth (1972) interpreted these traits which are common in species of *Tacca* as sapromyiopholous syndrome. There have not been detailed research regarding the reproductive biology and the mating system of *T. leontopetaloides*. However, Zhang et al. (2007) reported that outcrossing in *Tacca* may be rare. Cytological studies on T. *leontopetaloides* reported a chromosome number (2n = 30) (Darlington & Wylie 1955).

1.2.3. Other species of the genus *Tacca*

The genus *Tacca* has a pantropical rage with the main centre in Indo-Malaysia (Drenth 1972). Species of this small genus has diverse floral display and can generally be sorted into three groups based on their inflorescences characters according to Zhang et al. (2011): (i) *T.*

leontopetaloides, *T. plantaginea* and *T. parkeri* have inconspicuous inflorescences with small bracts and short bracteoles, (ii) *T. palmata* and *T. palmatifida* also have inconspicuous inflorescences with bracts but without bracteoles, and (iii) *T. subflabellata*, *T. integrifolia*, *T. ampliplacenta*, and *T. chantrieri* however, have very showy inflorescences with large bracts and long bracteoles.

Species in the *Tacca* genus produce no nectar and only a small amount of pollen (Zhang et al. 2005). *Tacca palmata and T. palmatifida* seem to lack any attraction to pollinators which could be as a result of septal nectary loss in monocots driven by the loss of pollinators (Smets et al. 2000). *Tacca subflabellata*, *T. integrifolia*, *T. ampliplacenta*, and *T. chantrieri* have two conspicuous and large bracts, dark purple or white in colour, positioned above the dark purple flowers and long dangling filiform bracteoles. The elaborate inflorescence structures with an impression of decaying organic matter attracting flies to facilitate cross-pollination is a deception and yields no reward. (Faegri and Van Der Pijl 1971; Drenth 1972). *T. chantrieri*, and *T. subflabellata*, are self-pollinating and lack effective pollinators (Zhang et al. 2005).

1.2.4. Origin and geographic distributions of *Tacca leontopetaloides*

Tacca leontopetaloides is a native to Western Africa, South East Asia, and Australia. It is the only species in its genus with the largest area of distribution, ranging from Africa and Madagascar to tropical Asia, Australia and Polynesia (Ukpabi et al. 2009) and widely spread in tropical areas, either as a native plant or naturalized, from Africa, through Asia. The distribution of *T. leontopetaloides* is believed to have taken place through the dispersion of the seeds by seawater, birds, and cultivation of its edible rhizomes far beyond its natural area by man (Drenth 1972). Domestication of this species by the local inhabitants of the Pacific Island is known (Ukpabi et al. 2009; Ubwa et al. 2011). Although *T. leontopetaloides* is sometimes cultivated in throughout the tropics of Africa, Asia, Australia and Pacific Islands, it is widely distributed in the wild according to Plant Resources of South- East Asia (Ukpabi et al. 2009). Figure 1 illustrates the distribution map of *Tacca leontopetaloides*.

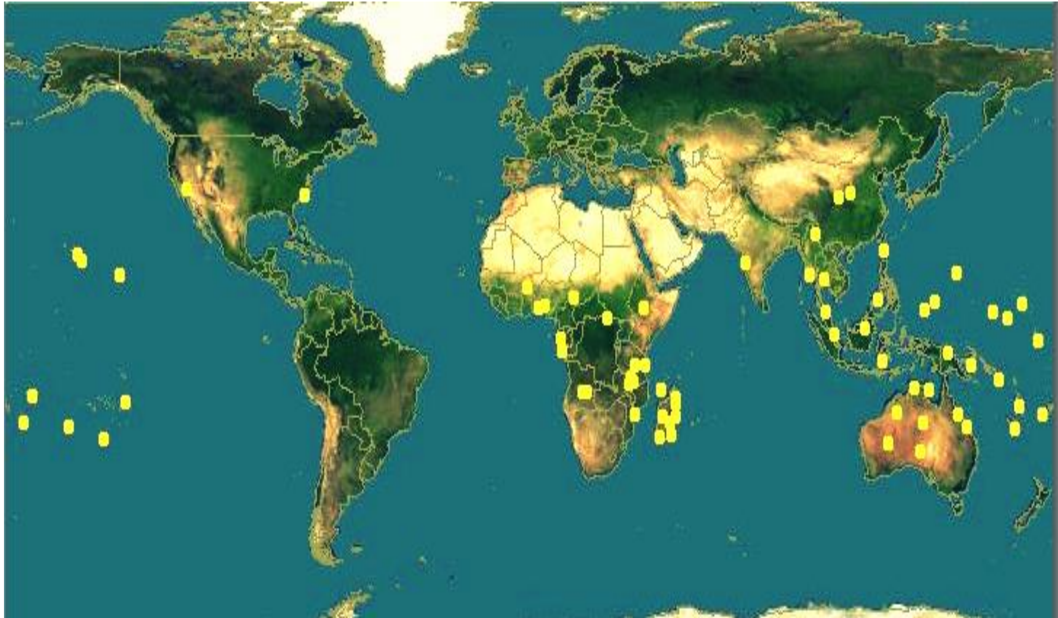

Fig. 1. Map showing the distribution of *Tacca leontopetaloides* Source: Wu et al. (2003)

1.2.5. Botanical description of *Tacca leontopetaloides*

1.2.5.1. Growth habit

The *Tacca* genus is exclusively composed of terrestrial, long-lived, stemless, rhizomatous herbs. Most species attain a maximum height of 50 cm to 100 cm. In *T. leontopetaloides* individuals of up to 3m high have been also found. The number of leaves and inflorescences in each plant is usually small (Drenth 1972). Figure 2 illustrates the growth habit of *Tacca leontopetaloides*.

Fig. 2. Growth habit *Tacca leontopetaloides*. Source: Wan Hong (2009)

1.2.5.2. Foliage

Tacca leontopetaloides leaves are characterized by herbaceous, hallow and erect petiole with a sheathing base. The base of the leaves and the inflorescence in young plants are surrounded cataphyll, a special, linear-lanceolate leaf. Each plant has about; 2-25 cm × 3.5 cm sheath; cylindrical, 17-150 cm long, 0.3-2.5 cm in diameter, light green, with white-green to blackish-purple dots, ridged longitudinally; blade broadly obovate, ovate or oblong-ovate in outline, up to 70 cm × 120 cm, glabrous, palmately 3-sect, with the 3 segments lobed dissected into orbicular to linear lobes. In the Pacific Islands, the radical leaves in *T. leontopetaloides* die off between December and March during which the plant usually remains dormant and new leaves will arise from the round underground tuber (Drenth 1972). In Nigeria, the plants are found mainly in the rainy season (March to August), while dormant through the dry season (from September to February) (Borokini et al. 2014). Figure 3 shows *T. leontopetaloides* leaves.

Fig. 3. *Tacca leontopetaloides* leaves. Source: Biechele (2013)

1.2.5.3. Inflorescence

Tacca leontopetaloides has 1 or 2 inflorescences per plant, borne on a leafless, unbranched scape arising directly from the rhizome/crown, usually cymose, but appearing umbel-like, surrounded by large, ovate involucral bracts in two whorls. The flowers are two whorls of three tepals, basally fused, green to dark brown or purple in colour, sometimes slightly fleshy (Caddick et al. 2002). The ovary is inferior, six-ribbed, unilocular, with three parietal placentas, and numerous ovules. In addition, the style is three-bilobed with broadened stigmatic branches, forming an umbrella-like structure, papillose on the underside. The base of style is often broadened and covered with multicellular glandular hairs. Figure 4 illustrates the inflorescence of *Tacca leontopetaloides*.

Fig. 4. Inflorescence of *Tacca leontopetaloides.* Source: Schmidt (2009)

1.2.5.4. Fruits and seeds

Fruits are subglobose, ovoid or ellipsoid, berry-like, up to 3.5 cm × 1.5-2.5 cm, pendulous, pale orange, and many-seeded. They are usually irregularly disintegrating, occasionally dehiscent. Seed are flattened, ovoid to an ellipsoid of about 5-8 mm × 3-5 mm × 1.5-3 mm glabrous, yellow-brown, but surrounded by a spongy white aril with 15-19-ribbed (Drenth 1972). Figure 5 illustrates the fruits and seed of *Tacca leontopetaloides*.

Fig. 5. A- Fruits and B-seed of *Tacca leontopetaloides*. Source: Biechele (2013)

1.2.5.5. Rhizomes and tubers

The tuberous rhizomes of *Tacca leontopetaloides* can be depressed globose or broadly ellipsoidal, up to 20 cm in diameter, weighing up to 0.9 kg, usually smaller and lighter, thin-skinned, smooth, white when young, turning dark grey to brown, white and somewhat juicy within, growing near the surface up to 50 cm deep, renewed annually, provided with an apical cavity from which the leaves and inflorescences emerge according to Plant Resources of South- East Asia (Drenth 1972). Figure 6 illustrates the tuberous rhizome of *T. leontopetaloides*.

Fig. 6. Tuberous rhizome of *Tacca leontopetaloides*. Source: Rulkens (2012)

1.2.6. Uses, importance and chemical composition of *Tacca leontopetaloides*

1.2.6.1. Uses and importance of *Tacca leontopetaloides*

Tacca leontopetaloides serves as an important food source. Tubers of these species were used as a famine food in the Marshall Islands during the twentieth century (Stone 1951; Kay 1987). It used to be a major source of carbohydrate in the savannah belt of Nigeria (Nwokocha et al. 2011).

The starch from Polynesian arrowroot is used a thickener in many dishes (Poyer 1990). It is also known that the starch can also be used to make alcoholic beverages (Doty 1954). Apart from the use of *T. leontopetaloides* starch as a source of carbohydrate, it has the potential to be used as a laundry starch (MacKenzie 1956; Pollock 1970). Harvested tubers are peeled, grated, washed several times in hot or cold water, and after the starch has settled, the water is removed and the starch dried (Spennemann 1994).

The fibre obtained from breaking the flower stalk was also used to make hats (Safford 1905). In recent times, researchers have also identified the Polynesian arrowroot tubers as a potential raw material source for biodegradable plastics production (Makhtar et al. 2013).

Tacca starch could also be useful for the pharmaceutical industry as a raw material for tablets formulation since it has similar physiochemical properties to maize starch (Kunle et al. 2003).

This species also has medicinal properties. The tubers are eaten raw for the treatment of diarrhoea and dysentery (Kay 1987). *T. leontopetaloides* has been over-collected for its medicinal properties resulting to local depletion in India (Bagul & Yadav 2007). Snakebite and some other ailments are treated in the Plateau state of Nigeria with *Tacca* root preparation (Borokini et al. 2012).

1.2.6.2. Chemical composition of *Tacca leontopetaloides*

Tacca leontopetaloides is cultivated or collected from the wild for its tubers and foliage which are used as a source of food or for medicinal purposes. The tuber has about 25% starch content (Ukpabi et al. 2009). However, the starch content usually varies ranging from10% to 25% of tuber weight depending on the growing conditions and soil substrate but Polynesian arrowroot starch is believed to be the richest natural starch (Ukpabi et al. 2009).

The amylose content in *T. leontopetaloides* starch is higher than that of maize but a lower content than potato starch. (Kunle et al. 2003). The flour of *T. leontopetaloides* tuber has following composition: water; 18.0%, fiber; 0.05%, total nitrogen; 0.01%, and ether extractives; 3.0% and the presence of sitosterol, ceryl alcohol and taccallin (0.003%), also gives positive tests for alkaloids (Peters et al. 1960).

There is the presence of tannins, cyanides, saponins and, flavonoids in small quantities while phytates, oxalates and alkaloids are present in appreciable amounts (Borokini & Ayodele 2012; Ogbonna et al. 2017).

Tannins may result to a reduction in digestibility and availability of the macromolecules thereby inhibiting microbial growth due to its ability to proteins and carbohydrates (Nwogu et al. 2008)

There are reports on flavonoids as a strong antioxidant (Sanjay & Rajendra 2015). They help in preventing the damage caused by free radicals to human cells. These compounds slow the proliferation of cancer cells, thereby mediating in most chronic cancer and diabetes cases (Ubwa et al. 2011). Flavonoids have also been shown to be able to affect various biological functions: capillary permeability, cellular secretory processes involved in the inflammatory response and, inhibition of enzymes, receptors and carriers (Gemede et al. 2014).

1.2.7. Cultivation and the stages of development of *Tacca leontopetaloides*

Tacca leontopetaloides grows in secondary forest and thickets, and many open situations, clearings, grassland, savannah, coconut groves (Spennemann 1994), and beach vegetation, as well as seasonally dry areas, such as teak and eucalypt woodland. It is a species of low elevations in the moist tropics, where it is most commonly found near the sea and below elevations of 200 metres (Kay 1987). It grows best in a fertile, humus-rich soil in the shade of trees but does not prefer saline soils (Stone 1951). *T. leontopetaloides* exhibits a seasonal growth rhythm. During the growing season, the tuber is replaced by a new main tuber which arises from a downward-growing runner-like thick rhizome at a lower level and remains dormant after a yearly death of the aerial parts of the original plant until the new growing season (Stone 1951). Secondary smaller runners, also forming tubers, may emerge above the old tuber and spread downwards. This cycle takes about 8-10 months, with 2-4 months of dormancy (Kay 1987). In Malaysia, flowering and fruiting may occur in all months of the year, but the aerial parts usually die off between December and March according to Plant Resources of South East Asia (Ukpabi et al. 2009). It is not known whether a plant flowers more than once during the vegetative period, but older plants have

relatively larger vegetative and generative parts and plants can set seed three years from being a seedling (Kay 1987).

T. leontopetaloides can be propagated by seed and by tuber. Usually, small secondary tubers left in the soil during harvesting act as seedlings for propagation at the beginning of the planting season (Stone 1951). Planting is done 15 cm deep, in rows at a spacing of 75 × 45 cm, preferably at the beginning of the rainy season (Paul 1965). It is reported that the crop benefits greatly from weeding (Stone 1951; Sproat 1968) and partial shade, and no serious diseases or pests are known. This species is a very hardy plant that can withstand droughts relatively well. In case of a severe drought, the top leafy part of the plant may die off, but the tubers survive and send up new shoots with the return of moisture (Soucie 1983). When the leaves begin to wither the tubers can be harvested by digging them up (Kay 1987). Individual tubers normally weigh from 70 to 340g but can reach 1 kg and two distinct forms have been reported from the Pacific Islands, one producing a single large tuber, the other with a number of smaller (potato-sized) tubers (Kay 1987). They may be stored in pits for later use but are liable to sprouting (Ukpabi et al. 2009).

1.2.8. Genetic variation and breeding of *Tacca leontopetaloides*

Neither germplasm collections nor breeding programmes are known to exist for *T. leontopetaloides*. Sproat (1968) reported an observation for two varieties of *T. leontopetaloides* from atolls in Micronesia. However, Spennemann (1994) reported four varieties or subvarieties of *T. leontopetaloides* recognized by the Marshallese which are not separately named. He reported that one possesses violet-purple leaf stalks with brown skin and yellow to white interior tubers whiles the other three varieties possess green stems and stalks with one of these producing a single large tuber and the other two producing more than one tuber.

Germplasm collection is urgently recommended, as in many places its natural habitat is being rapidly destroyed and its use for medicinal purposes resulting to local depletion (Bagul and Yadav 2007).

1.2.9. Polyploidization and its use in plant breeding

Generally, polyploidy is defined as the possession of three or more complete copies of the nuclear chromosome set. Polyploidy in plants is believed to have been discovered a century ago and is considered to be an important feature of chromosome evolution (Ramsey & Schemske 1998). There is a widespread of occurrence of polyploidy in natural populations (Wood et al. 2009) suggesting its major evolutionary force driving both speciation and diversification (Otto & Whitton 2000; Soltis et al. 2009) as opposed to the longtime view of polyploidy as an evolutionary dead end. Polyploids have a high level of genomic plasticity and this provides an evolutionary advantage over their diploid complements due to excess of genomic material in polyploids (Hegarty & Hiscock 2008; Leitch & Leitch 2008; Van de Peer et al. 2009).

Improvement of many plant species and hybrids resulted from the important roles played by artificial induction of polyploidy resulting in traits in which many breeders have selected and used as parents (Paulo et al. 2000). Tetraploids obtained from interspecific crosses of *Manihot epruinosa* (2n=36) and *Manihot glaziovii* (2n=36) performed as well as the best variety in uniform yield (Hahn et al. 1990). Viehmannová et al. (2009) reported that Fructooligosaccharides (FOS) content in the tuberous roots can be increased by polyploidy breeding according to the results obtained from hexadecaploid yacon tubers.

Polyploids can arise spontaneously within plants during somatic cell division (mitosis) which can result in an autopolyploid shoot often noticeable by its enlarged condition (Stebbins 1971). Other methods of inducing polyploids include treatments with mitotic inhibiting chemicals such as colchicine (Derman 1940), oryzalin (Dolezel et al. 1994), trifluralin (Eeckhaut et al. 2004) and amiprophos- methyl (Hansen et al. 2000)

Colchicine is an alkaloid extracted from seeds or corms of *Colchicum autumnale* L. (autumn crocus or meadow saffron) and was first isolated in 1820 by the French chemists P.S. Pelletier and J. Caventon (Pelletier & Caventon 1820). Ploidy levels have been manipulated in plants (Derman 1940) by submerging the specimen in a solution of colchicine.

The active ingredient of the pre-emergence herbicide Surflan® which is Oryzalin [3, 5-dinitro-N4, N4-dipropylsulfanilamide] (Sourthern 1998) is also used to induce polyploidy in plants. The ploidy

level of several plant species has been altered using oryzalin. Some of such species include *Miscanthus sinensis* Anderson (Petersen et al. 2002) and *Tulipa gesneriana* L. (Chauvin et al. 2005). Research with *Miscanthus sinensis* determined that treating shoot apices in 15 μM oryzalin solution for a period of 96 hours was the most effective treatment for inducing polyploids (Petersen et al. 2002). In *Solanum tuberosum* L. the most effective treatment for producing tetraploids was a 24 hours treatment with 28.8 μM oryzalin solution applied to apical buds (Chauvin et al. 2003).These research indicate that the optimal oryzalin concentration and treatment duration for polyploid induction varies among species and must be determined empirically. Oryzalin and trifluralin are often more effective than colchicine because they have a higher affinity for plant tubulins (Dolezel et al. 1994).

The effectiveness of these compounds *in vitro* depends highly on the concentration applied, duration of treatment, type of explant, and the penetration of the compound (Allum et al. 2007). In many instances, oryzalin and trifluralin are more effective at stable ploidy induction, have an increased survival of explants, and are used at lower concentrations than colchicine (Ganga & Chezhiyan 2002; Zlesak et al. 2005).

Flow-cytometry is used to analyze and quantify nuclei DNA content for polyploidy detection. The evaluation of a large population of single cells or nuclei by quantifying the amount of nuclear DNA that is present can be done using a flow cytometer. This is done by aligning cells or nuclei, via hydrodynamic forcing, and passing them by a single wavelength light source (Greve et al. 2004). When a DNA fluorescent stain such as 4´, 6-diamidino-2-phenylindole (DAPI) is included the light excites the bound DAPI and emits fluorescence proportional to the DNA/ DAPI binding ratio. This emission is in the form of an electrical signal that is translated into a numerical data set that is compiled in real time (Rahman 2006). Polyploidy can also be detected by chromosome counting. During meiotic or mitotic cell division the chromosome number can be established in cells. Counting of mitotic chromosomes is easier and faster and root tips are known to be the most convenient source of mitotic cells but when roots are no available, young buds, leaves or callus can be used (Maluszynska 2003).

1.2.10. Micropropagation of *Tacca leontopetaloides*

Propagation of plants under *in vitro* conditions has over the years been successful in the field of plants breeding and improvement in crop production. Micropropagation offers an alternative method of vegetative propagation for mass crop propagation. Growth and vigour of tuber crops have been reported to have been increased from *in vitro* propagation using Murashige and Skoog (MS) medium (1962) (Hussey and Stacey 1981). Using Murashige and Skoog (MS) medium (1962) supplemented with plant growth regulators (PGRs) has been proven to effectively induce shoot and root formation of tuber crops *in vitro*. According to Kohmura et al. (1995), MS medium supplemented with α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) effectively induced shoot formation leading to plantlet formation in 'Yamatoimo' Chinese yam (*Dioscorea opposita* Thunb.). Poornima and Ravishankar (2007) also reported *in vitro* propagation of two wild yams, *Dioscorea oppositifolia* and *Dioscorea pentaphylla* where multiple shoots were initiated from nodal explants on Murashige and Skoog (MS) medium supplemented with 8.8 µM BAP and 0.3% (w/v) activated charcoal.

Micropropagation of *T. leontopetaloides* has also gained the attention of crop scientist and plant breeders due to the importance of the species as a potential source of starch, its medicinal properties as well its potential for food security. Micropropagation would be useful for the multiplication of initial plant materials of *T. leontopetaloides* to obtain sufficient plant materials for this research and to multiply the new polyploidy genotypes. Hlasna Cepkova et al. (2015) developed a simplified micropropagation protocol and optimized MS salts and 0.05 mg l^{-1} NAA in combination with 0.1 mg l^{-1} zeatin for both propagation and rooting of *T. leontopetaloides*.

2. Aims of the thesis

The aim of the thesis was to induce *in vitro* polyploidization using oryzalin as an antimicrotubule agent, to detect polyploids using flow cytometry and to assess polyploids *in vitro* and in field conditions under the tropical conditions of Ghana.

The partial aims of the thesis were following:

- Initial *in vitro* propagation of diploid plants for the purposes of the polyploidization experiment.
- Induction of polyploidy in *T. leontopetaloides* using oryzalin at various concentrations and time durations.
- Detection of polyploid plants using flow-cytometry
- Evaluation of the morphology and growth characteristics of polyploidy genotype under *in vitro* condition.
- *Ex vitro* transfer and evaluation of polyploid genotype and diploid plants under greenhouse conditions.
- Evaluation of polyploid genotype plants under the field conditions in Ghana to evaluate plant morphology and the tuber characteristics of *T. leontopetaloides*.

The objectives of the diploma thesis were set under the following hypothesis:

- Oryzalin as efficient antimicrotubule agent induces polyploidization in various plant species. It is hypothesized that its application will double chromosome count in *T. leontopetaloides.*
- Induction of polyploidy using oryzalin is able to alter the morphological and yield characteristics of *T. leontopetaloides* plants compared to the original plant materials from which they were obtained.
- The polyploids obtained will show some improved desirable traits.

3. Material and methods

3.1. Research collaboration institutions

Polyploidization, detection of polyploidy as well as morphological and growth *in vitro* evaluation of polyploids were carried out at the Czech University of Life Sciences Prague (CULS), Czech Republic.

Ex vitro transfer and field evaluation of polyploid plants were carried at the University of Cape Coast, Ghana.

3.2. Plant material

The initial *T. leontopetaloides* diploid (2n = 30) plant material was obtained from a previous study carried out by Hlasna Cepkova et al. (2015), where a simplified micropropagation protocol was developed for *T. leontopetaloides*. Hlasna Cepkova et al. (2015) established *in vitro* culture of *T. leontopetaloides* from seeds obtained from the Botanical Garden of the Faculty of Tropical AgriSciences, CULS. The plants were kept under *in vitro* conditions on full strength MS medium without PGRs at 25/20 °C and maintained in a growth chamber with 16/8 h light/dark regime, and at a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1}, provided by cool-white fluorescent tubes. Figure 7 illustrates the initial plant material for this research. Figure 7 illustrates the initial plant material used for the research.

Fig. 7. Initial plant material of *T. leontopetaloides* used for the research. Source: Author

3.3. Methods

3.3.1. Multiplication of plant material

For initial multiplication of this material, 0.3-0.5 cm long shoots growing from the basal part of the explant, were propagated in 100 ml Erlenmeyer flasks containing 30 ml of MS medium without PGRs with the addition of 100 mg l^{-1} *myo*-inositol, 30 g l^{-1} sucrose and 8 g l^{-1} agar at a pH of 5.7. The culture medium was sterilized in an autoclave at 120 °C and a pressure of 100 Pa for 20 min. The plants were regularly sub cultivated every 28 days on the same medium to obtain sufficient plant material for the experiment.

3.3.2. Induction of polyploidy in *Tacca leontopetaloides*

A total of 120 shoots each of 0.3-0.5 cm long growing from the basal part of the explant were used for the experiment. A total of six 1 litre volumetric flasks each containing 20 explants cultured on 30 ml of MS medium without PGRs supplemented with 100 mg l^{-1} myo-inositol and 30 g l^{-1} sucrose at pH 5.7 for two days were used. Two treatments comprising the use of sterile distilled

water (control treatment) for 24 or 48 hours exposure time and the use of oryzalin of two levels of concentration (25 µM and 30 µM) for 24 or 48 hours exposure time for each concentration level. Oryzalin solution was prepared by dissolving 0.0346 g of oryzalin in 10 ml of dimethylsulfoxide (DMSO) in a flow box. This sterilized the oryzalin and also help it to penetrate through cell walls of the explants. One hundred millilitres each of 25 µM and 30 µM oryzalin solutions containing 2% (DMSO) as well as sterile distilled water were used to soak the explants for 24 or 48 h for the respective treatments. The treated shoots were rinsed using sterile distilled water three times after the oryzalin treatment and placed individually on a fresh MS medium without PGRs. Cultures were maintained at 25/20 °C under a 16/8 h light/dark regime with 36 µmol m^{-2} s^{-1} cool white fluorescent light. Shoots regenerated were cut off and transferred to MS medium without PGRs, where they rooted spontaneously. Control plants and the oryzalin treated plants were sustainably maintained with regular subculture on MS medium for 40 days and subjected to flow cytometric analysis.

3.3.3. Detection of *Tacca leontopetaloides* polyploids using flow cytometry

Cytometric analysis was done using a CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Sample preparation was done by employing the two-step methodology according to Dolezel et al. (2007). *Glycine max* cv. Polanka, 2C = 2.50 pg; (Dolezel et al. 1994) was used as internal reference standard. Using razor a blade, about 0.5 cm^2 of *T. leontopetaloides* leaf *in vitro* sample and an appropriate amount of internal reference standard were chopped in 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% (v/v) Tween 20). The suspension of nuclei was filtered using 0.42 µm nylon mesh. The filtrate was thereafter incubated for about 10 minutes at room temperature and stained with a solution consisting of 1 ml of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) supplemented by AT-selective fluorescent dye DAPI (4,6-diamino-2-phenylindol) and 2-mercaptoethanol in final concentrations of 4 µg ml^{-1} and 2 µl ml^{-1}, respectively. The stained nuclei after incubation of the sample at room temperature for about 3-4 minutes were analyzed with a flow cytometer at a concentration of 2,000 per sample. FloMax software (ver. 2.4d; Partec GmbH, Münster, Germany) was used to evaluate histograms obtained from the cytometry analysis. Ploidy level was measured in all plants treated with oryzalin Ploidy stability was controlled by repeated flow cytometry analysis of plantlets after each subculture.

3.3.4. *In vitro* morphological and growth evaluation of polyploid genotype

3.3.4.1. Morphological evaluation using MS medium without PGRs

The morphological evaluation of *T. leontopetaloides* diploid and polypoid genotype was done using MS medium without PGRs. One hundred millilitres Erlenmeyer flasks containing 30 ml of medium were used to culture the explants. A total of 40 explants were used for the experiment comprising 20 diploid and 20 polyploid genotype explants in two replications. The cultures were placed in cultivation room at 25/20 °C under a 16/8 h light/dark regime, and at a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1}, provided by cool-white fluorescent tubes.Growth habit, the shape of the leaf as well as leaf colouration were observed. Ten fully developed leaves each from diploid and tetraploid were isolated and the width and the length of the leaf blades measured to determine the differences in leaf size. The shape of the leaves was also compared by isolating the youngest and oldest leaf for diploid and tetraploid after 8 weeks of *in vitro* cultivation. The height of shoots, number of shoots, number of leaves, and number of roots, as well as the length of roots were evaluated after 8 weeks of *in vitro* cultivation.

3.3.4.2. Growth evaluation using optimal growth medium

The growth of *T. leontopetaloides* diploid and polyploidy plants was evaluated using the optimal growth medium for *in vitro Tacca* propagation. This medium was optimized by Hlasna Cepkova et al. (2015) and it consisted of MS salts and 0.05 mg l^{-1} NAA in combination with 0.1 mg l^{-1} zeatin. Thirty millilitres of the medium in 100 ml Erlenmeyer flasks were used to culture the explants. A total of 180 explants were used for the experiment comprising 45 diploid and 45 polyploid explants in two replications. The cultures were placed in cultivation room at 25/20 °C under a 16/8 h light/dark regime, and at a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1}, provided by cool-white fluorescent tubes. The height of shoots, number of shoots, number of leaves, the number of roots and length of roots were measured after 8 weeks of *in vitro* cultivation.

3.3.5. *Ex vitro* transfer

In total, 24 well-rooted plants comprising 12 diploid and 12 polyploidy genotype obtained after 8 weeks of the subculture of explants on the medium containing 0.1 mg l^{-1} zeatin and 0.05 mg l^{-1} NAA were removed from Erlenmeyer flasks. All traces of any adhering medium were removed by rinsing the roots with tap water. The plants were subsequently transplanted to 10 cm plastic nursery bags containing a sterilized planting medium consisting of sand, soil and organic material (3:2:1). The cultures were watered and covered with transparent polyethylene foil to maintain high air humidity (80-90%), temperature (ca. 30/23 °C day/night), and placed in a greenhouse for acclimatization. After 3 weeks, the foil was gradually removed; from the 4th week, the plants were exposed to greenhouse culture conditions (70-87% air humidity, the temperature ca. 28/20 °C day/night). The number of shoots and height of plants was evaluated on the 2nd, 4th, and the 6th week under greenhouse condition. The survival rate of the plants was recorded 6 weeks after *ex vitro* transfer.

3.3.6. Transplantation of *T. leontopetaloides* plants to field conditions

After 6 weeks of greenhouse cultivation, the plants were transplanted to the experimental plots. Ten plants each from the diploid and polyploid plants were established in furrows 15 cm deep, in rows at a spacing of 75 × 45 cm, (Paul 1965). Plants of each genotype were labelled and numbered from 1-10. Field transplantation was carried out in the month of November at the University of Cape Coast located in the coastal area in the central region of Ghana. The cultivation area has the least amount of rainfall occurring from the month of December to February with an average of 24 mm. The precipitation reaches its peak in the month of June with an average of 327 mm. Temperatures are high throughout the year with an average 34/26 °C day/night. The average annual humidity for this area is 77%. No irrigation was carried out during the entire vegetation period. Field observation was carried out to access the growth habit, development of shoots and survival rate of the diploid and polyploidy genotype on the field.

3.3.7. Harvesting and evaluation of *Tacca leontopetaloides* microtubers

Microtubers were harvested after 12 weeks of field cultivation. Microtubers were removed from the soil by hand, washed under tap water to remove the dirt and labelled. Primary evaluation of microtubers was carried out to assess the number of tubers per plant, the weight of tubers and the average diameter of the tubers for diploid and tetraploid plants.

3.3.8. Experiment design and statistical evaluation

A completely randomized design for *in vitro* experiments was set up. The data obtained from the micropropagation experiment was statistically analysed using one-way ANOVA, and the significant differences between means were assessed by Tukey's HSD test at the 5% level of significance ($P \leq 0.05$) (STATISTICA 12.0, StatSoft).

4. Results

4.1. Induction and detection of polyploidy in *T. leontopetaloides*

After 14 days of *in vitro* cultivation of oryzalin treated explants on MS medium, plants started to regenerate from the original explant. In total seven of 80 (8.75%) plantlets treated with oryzalin were polyploid. Table 1 indicates that the highest polyploidization efficiency of 10% was achieved with 25 µM oryzalin regardless of time duration and with 30 µM for 24 hours exposure time. Overall, the survival rate in plants decreased with both time duration and concentration of oryzalin. The survival rate was highest for 25 µM at 24 hours except for control treatment. Longer exposure time (48 h) of oryzalin at concentration 30 µM resulted in lower survival rate (55%) as well as polyploidization efficiency (5%). No mixoploids, containing diploid and tetraploid cells, were detected. Polyploids were detected using flow cytometry.

Histograms showed two peaks. The first peak represented nuclei in the G0/G1 phase of the cell cycle belonging to *T. leontopetaloides* sample and the second peak corresponded to nuclei of the internal standard (*Glycine max*) in the G0/G1 phase (Figure 8). The DNA-ratios of *in vitro* regenerants of *T. leontopetaloides* varied from 0.376-0.3790 in diploid and from 0.727-0.754 in polyploidy regenerants, indicating that the genome has been doubled during polyploidization.

Table 1. *In vitro* oryzalin treatment effect on induction of polyploidy in *T. leontopetaloides*

Oryzalin concentration (µM)	Exposure time (h)	Number of explants (shoots)	Survival rate (%)	Tetraploids detected by flow cytometry	Polyploidization efficiency (%)	Mixoploid plants (%)
0 µM (control)	24	20	100	0	0.0	0
0 µM (control)	48	20	100	0	0.0	0
25 µM	24	20	90	2	10.0	0
25 µM	48	20	65	2	10.0	0
30 µM	24	20	75	2	10.0	0
30 µM	48	20	55	1	5.0	0

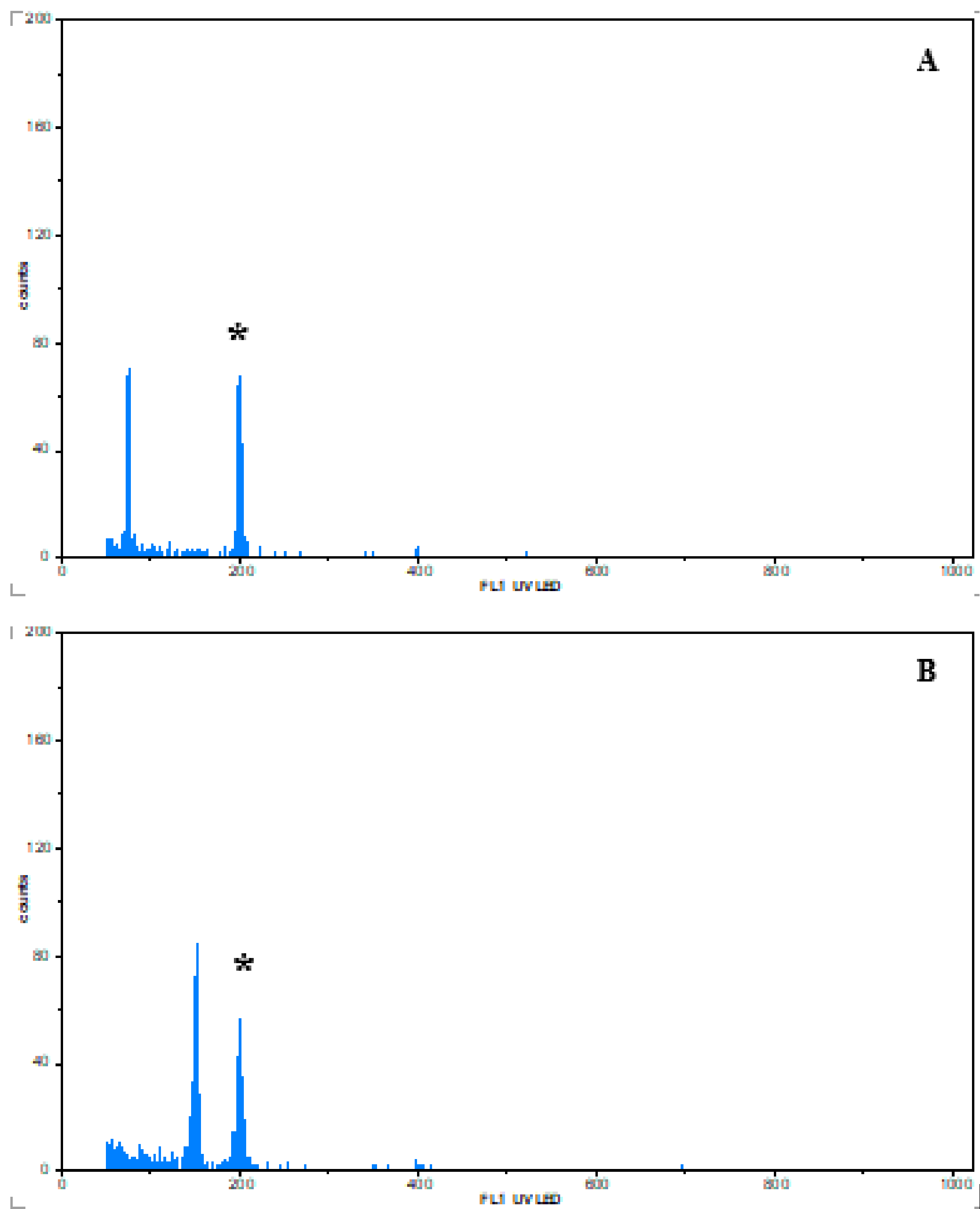

Fig. 8. Flow cytometric histograms of DAPI stained nuclei of an *in vitro* regenerated *T. leontopetaloides* (A) diploid plant and (B) tetraploid plant. Peak indicated as "*" corresponds to the internal reference standard *Glycine max*.

4.2. *In vitro* evaluation of *T. leontopetaloides* diploid and tetraploid

4.2.1. Morphological evaluation using MS medium without PGRs

Morphological characteristics of *T. leontopetaloides* diploid and tetraploid were evaluated on MS medium without PGRs. Diploid and tetraploid shoots began to regenerate after one week of *in vitro* cultivation. Tetraploid showed thicker shoots. Results showed no significant difference in plant height. Roots grew at a slower rate in diploid in comparison to tetraploid. The results showed significantly higher number of shoots for diploid plants in comparison to tetraploids. Number of roots and the length of roots were significantly higher for tetraploid plants (Table 2). Visual observation also indicate a thicker roots for tetraploid (Figure 9).

Table 2. Morphological evaluation of *T. leontopetaloides* diploid and tetraploid plants using MS medium without PGRs

Treatment		**Mean ± S.E.**	
	Growth Characteristics	**Control plants**	**Polyploid plants**
MS medium without PGRs	Height of plant (cm)	2.54 ± 0.20^{a}	2.41 ± 0.20^{a}
	Number of shoots per plant	6.35 ± 0.82^{a}	3.80 ± 0.51^{b}
	Number of roots per plant	2.95 ± 0.52^{b}	4.85 ± 0.66^{a}
	Length of root (cm)	0.62 ± 0.10^{b}	0.91 ± 0.10^{a}

Means with different superscript letters are significantly different according to Tukey's HSD test at the 5% level of significance ($P \leq 0.05$) *S.E. standard error

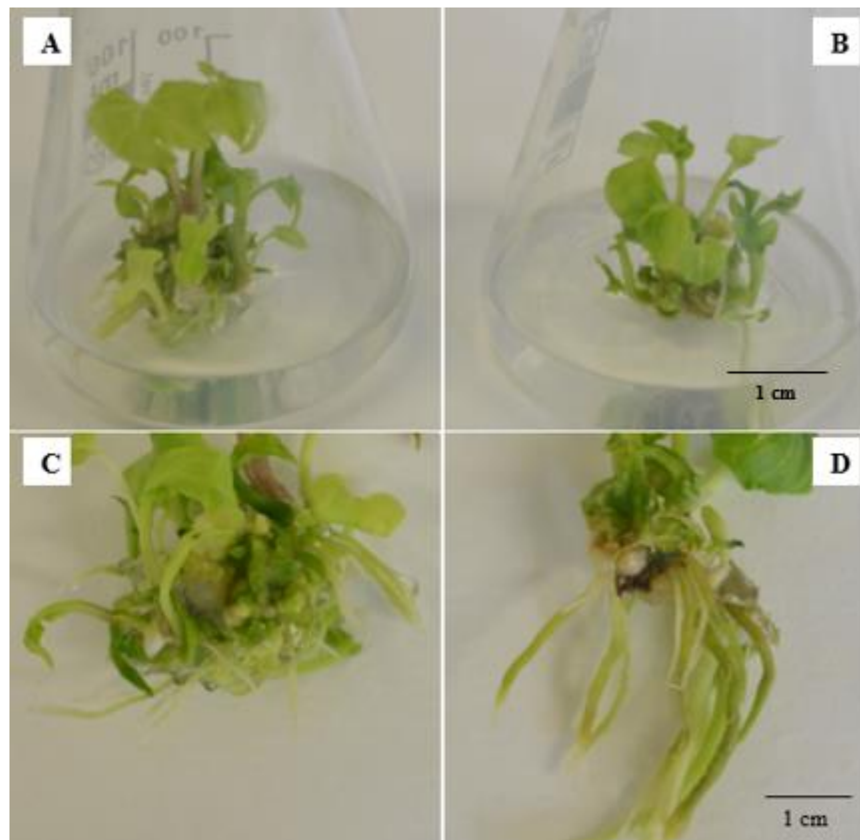

Fig. 9. Morphological evaluation of *T. leontopetaloides* (A-diploid, B-tetraploid) growth habit and (C-diploid, D-tetraploid) root development using MS medium without PGRs. Source: Author

Ten fully developed leaves each from diploid and tetraploid plants were isolated and the width and length of the leaf blades were measured after 8 weeks of *in vitro* culture. Results from leaf measurement of *T. leontopetaloides* diploid and tetraploid leaves indicated that tetraploid tend to have an increase in both leaf width and length as compared to diploid plants. An average width to length ratio of (1.55 ± 0.12) was recorded for tetraploid plants while diploid plants recorded (1.37 ± 0.06) with tetraploid leaves (Table 3). This indicates that tetraploid plants have a somewhat wider leaf in comparison to their diploid counterparts (Figure 10).

Table 3. Morphological assessment of *T. leontopetaloides* diploid and tetraploid leaves

Control plants			**Polyploid plants**		
width (cm)	**length (cm)**	**width/length ratio**	**width (cm)**	**length (cm)**	**width/length ratio**
2.0	1.4	1.43	2.2	1.7	1.29
2.1	1.5	1.40	2.2	2.3	0.96
2.2	1.8	1.22	2.9	1.6	1.81
1.6	1.4	1.14	2.6	1.9	1.37
1.9	1.5	1.26	2.3	1.3	1.78
1.8	1.2	1.50	1.6	1.4	1.14
1.5	1.2	1.25	2.5	1.6	1.56
1.6	1.3	1.23	2.9	1.3	2.23
1.9	1.2	1.58	2.8	1.5	1.87
1.5	0.9	1.67	1.9	1.3	1.46
1.81 ± 0.08*	1.34 ± 0.08*	1.37 ± 0.06*	2.39 ± 0.14*	1.59 ± 0.10*	1.55 ± 0.12*

*Mean ± S.E. (standard error)

Visual assessment of morphological difference of young and older leaves for diploid and tetraploid plants isolated after 8 weeks of *in vitro* cultivation showed a noticeable difference in leaf shape (Figure. 11). In diploid plants, older leaves are palmately lobed while young leaves are palmate in shape with incised lobes. Young and older leaves of tetraploid variants appeared rounder than diploid leaves. Similarly, leaves of tetraploid variants appeared thicker and slightly pigmented than diploid leaves.

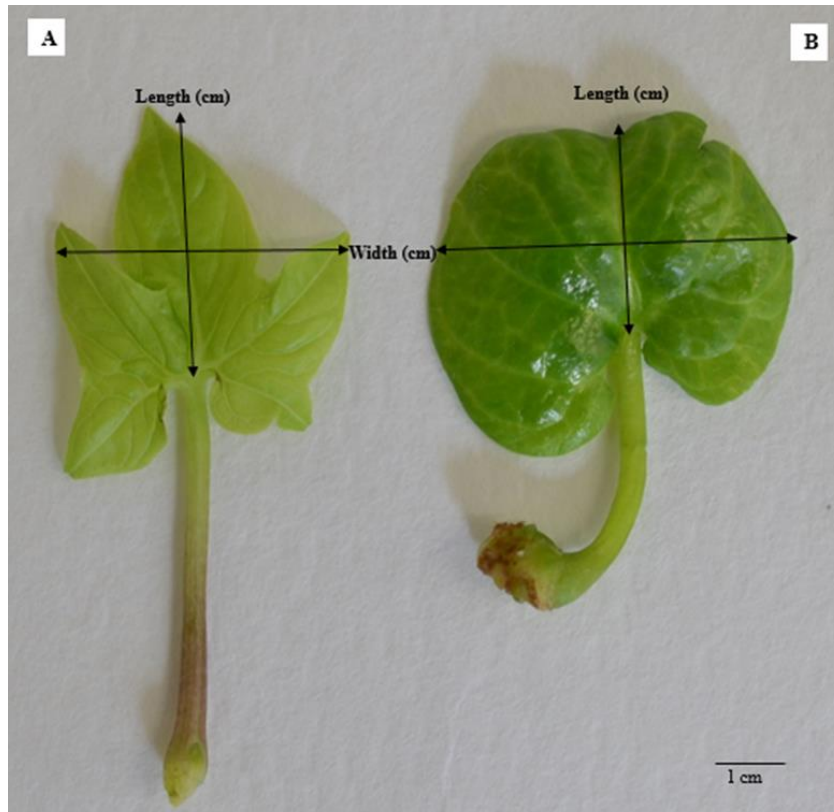

Fig. 10. Measurement of leaf sizes as part of the morphological evaluation *T. leontopetaloides* for (A) control (diploid) and (B) polyploid (tetraploid) plants. Source: Author

Fig. 11. Demonstration of shape of older and young leaves of *T. leontopetaloides* for (A) control (diploid) and (B) polyploid (tetraploid) plants. Source: Author

4.2.2. Growth evaluation of tetraploid

Growth evaluation of tetraploid was carried out on MS medium supplemented 0.1 mg l^{-1} zeatin and 0.05 mg l^{-1} NAA. Table 4 indicates that diploid plants are significantly different from tetraploid plants for all growth characteristics. Diploid plants showed significantly higher mean for plant height, number of shoots, and length of roots. The number of leaves corresponded to the numbers of shoots as each shoot produced a single leaf. However, tetraploid plants showed significantly higher mean value for the number of roots (Figure 12). Visual observation indicates thicker shoots and roots in tetraploid plants.

Table 4. Effect of MS medium supplemented with zeatin and NAA on growth characteristics of *T. leontopetaloides* diploid and tetraploid

Treatment		Mean ± S.E.	
	Growth Characteristics	**Control plants**	**Polyploid plants**
MS medium + 0.1 mg l^{-1} ZEA + 0.05 mg l^{-1} NAA	Height of plant (cm)	3.10 ± 0.11^{a}	2.25 ± 0.05^{b}
	Number of shoots per plant	5.06 ± 0.19^{a}	4.43 ± 0.16^{b}
	Number of roots per plant	4.04 ± 0.18^{b}	7.27 ± 0.19^{a}
	Length of root (cm)	1.32 ± 0.07^{a}	1.12 ± 0.03^{b}

Means with different superscript letters are significantly different according to Tukey's HSD test at the 5% level of significance ($P \leq 0.05$) *S.E. standard error

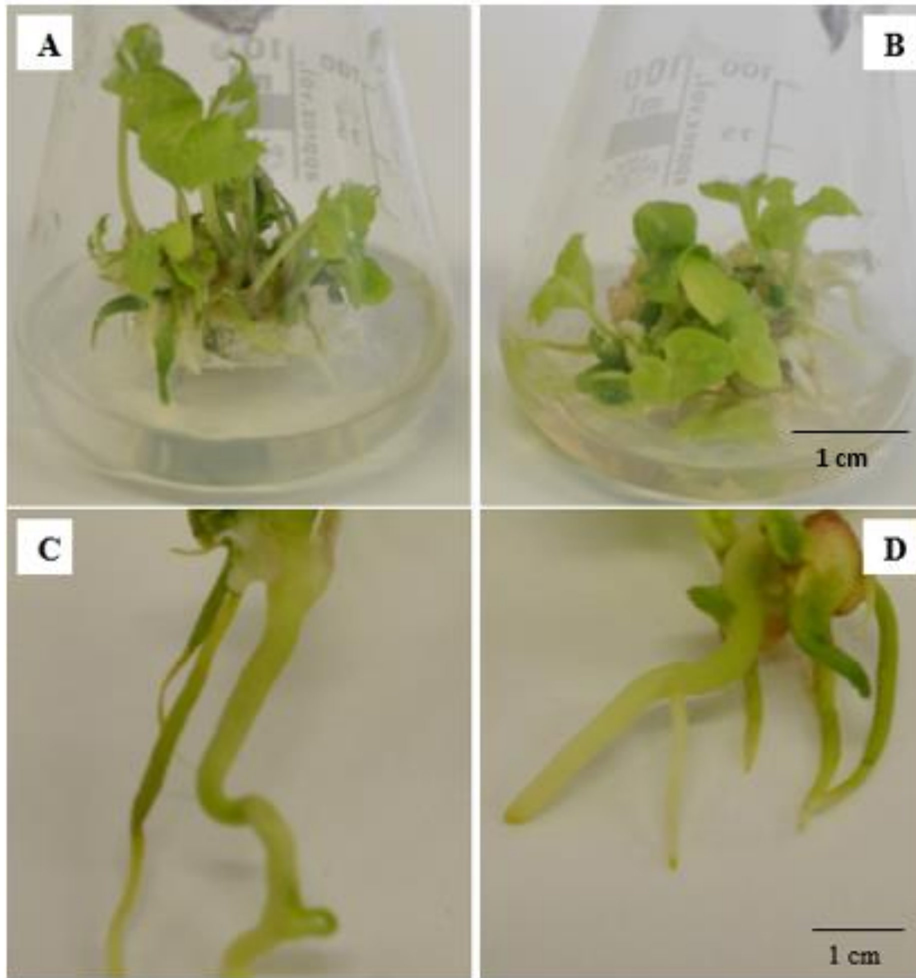

Fig. 12. Growth evaluation of *T. leontopetaloides* (A-diploid, B-tetraploid) growth habit and (C-diploid, D-tetraploid) root development using MS medium supplemented with 0.1 mg l^{-1} zeatin and 0.05 mg l^{-1} NAA. Source: Author

4.3. Evaluation of *T. leontopetaloides* plants after *ex vitro* transfer

Acclimatization of *T. leontopetaloides ex vitro* under greenhouse conditions showed an overall a higher survival rate of 100% and 91.6% after 6 weeks for diploid and tetraploid respectively. After 6 weeks of *ex vitro* transfer diploid plants recorded higher average plant height (9.4 ± 0.78 cm) as well as number of shoots (8.2 ± 1.00 shoots/plant) as compared to tetraploid with (8.6 ± 0.87 cm) plant height and (4.5 ± 0.80 shoots/plant) (Figure 13 and 14). Since the number of leaves corresponds to the number of shoots, diploid plants recorded also a higher number of leaves as compared to tetraploid plants. There were also noticeable differences in leaf shape and size for diploid and tetraploid (Figure 15). Visual observation indicates that tetraploid plants had larger leaf area with thicker leaf blade while diploid plants have pale green, palmately lobed and smaller leaf area.

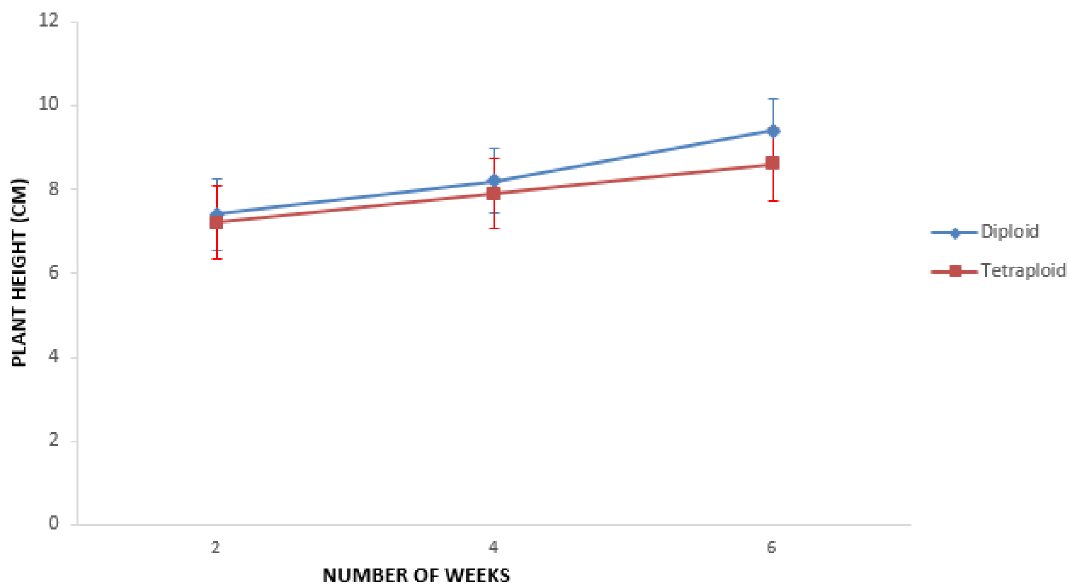

Fig. 13. Morphological evaluation of *T. leontopetaloides* diploid and tetraploid plants height over the period of 6 weeks under greenhouse conditions in Ghana. Source: Author

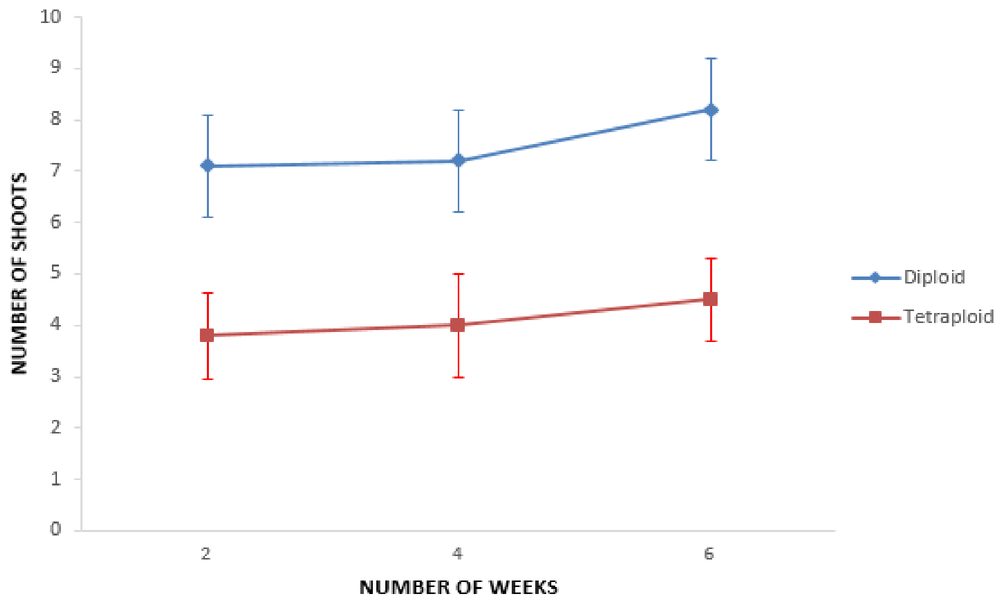

Fig. 14. Morphological evaluation of *T. leontopetaloides* of diploid and tetraploid number of shoots over the period of 6 weeks under greenhouse conditions in Ghana. Source: Author

Fig. 15. *Ex vitro* transfer of *T. leontopetaloides* (A) tetraploid and (B) diploid (control) plants. Source: Author

4.4. Field transplantation and observation of the growth and development of *T. leontopetaloides*

A total of 20 plants consisting of 10 diploid plants and 10 tetraploid were established on the field (Figure 16) in Ghana, at the University of Cape Coast, Cape Coast. There was no new shoots development for both diploid and tetraploid plants in field conditions. Plants grew at a steady rate with diploid plants showing a steady increase in plant height as compared to tetraploid plants. Tetraploids recorded 80% survival rate with 60% for diploid plants after 12 weeks of field cultivation. After eight weeks of field cultivation, leaves began to turn yellow (Figure 17) and the plants failed to develop. Shoots became weak and the aboveground part of the plants started to die.

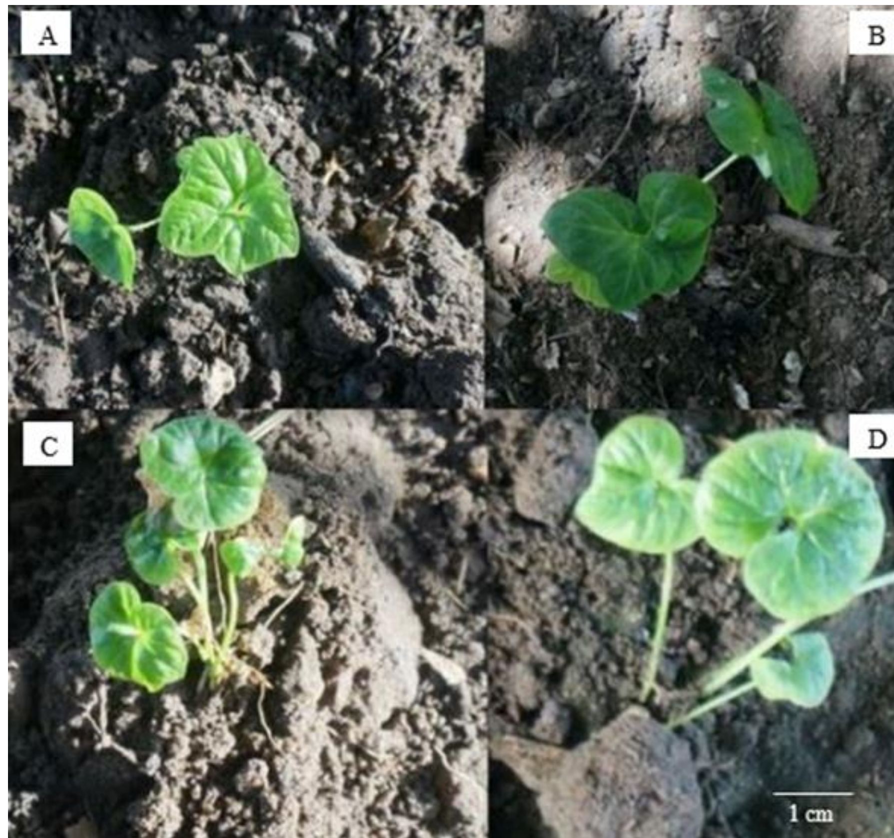

Fig. 16. Field transplantation of *T. leontopetaloides* diploid (A) 3 weeks (B) 6 weeks after field cultivation and tetraploid (C) 3 weeks (D) 6 weeks after field cultivation. Source: Author

Fig. 17. *Tacca leontopetaloides* plant on the field showing yellowing of leaves after 8 weeks in field cultivation. Source: Author

4.5. Harvesting and evaluation of *Tacca leontopetaloides* microtubers

Lodging of shoots and death of the aboveground part of both diploid and tetraploid plants were observed after 12 weeks of field cultivation. Microtubers were then harvested for primary evaluation (Figure 18). It was observed that only two plants form the survived diploid plants produced micro tuber with an average of (1.5 ± 0.50 tubers/plant) microtubers per plant while four plants from the survived tetraploid plants produced microtubers with an average of (2.5 ± 0.29 tubers/plant) microtubers per plant. Microtubers from diploid plants recorded a mean weight per tuber (1.78 ± 0.19 g) with a mean tuber diameter of (1.03 ± 0.09 cm) while tetraploid recorded a mean weight of (3.06 ± 0.16 g) with a mean microtuber diameter of (1.35 ± 0.10 cm). Visual observation indicates tetraploid have smooth and thin-skinned microtubers while diploid have rough and thicker-skinned microtubers (Figure 19).

Fig. 18. Harvested plants with tubers of *T. leontopetaloides* after 12 weeks of field cultivation (A) diploid, (B) tetraploid. Source: Author

Fig. 19. Visual observation of microtubers of *T. leontopetaloides* (A) diploid and (B) tetraploid after harvesting. Source: Author

5. Discussion

5.1. *In vitro* polyploidization

This study presents the first report of induction of polyploidization in *Tacca leontopetaloides*. In this research, it was proven that oryzalin is an appropriate antimitotic agent for *in vitro* polyploidization and chromosome doubling. Similar responses on the use of oryzalin for chromosome doubling have been reported in *Smallanthus sonchifolius* (Viehmannová et al.2009) where hexadecaploid from octaploid was induced, *Lilium davidii* var. *unicolor* (Feng et al. 2017) as well as in *Ullucus tuberosus* (Viehmannová et al. 2012). In this study, oryzalin treatment was most efficient (10%) for chromosome doubling at concentration of 25 µM irrespective of the exposure time (24 or 48 hours) and 30 µM for 24 hours exposure time. Although similar oryzalin concentration 25 µM in *Ullucus tuberosus* (Viehmannová et al. 2012), or 30-40 µM in *Chaenomeles japonica* (Stanys et al. 2006) have been report for efficient chromosome doubling, oryzalin treatment concentration appears to be species dependent since . Lower concentration of 1 µM for longer exposure time (10 weeks application in cultivation medium) was reported to be most efficient for chromosome doubling in *Anemone sylvestris* (Zahumenická et al. 2018) whereas Bouvier et al. (2002) reported 200–300 µM for 48 hours exposure time as the optimal oryzalin concentration in haploid polyploidization of pear. However, in our study, 30 µM for longer exposure time of 48 hours resulted in lower polyploidization efficiency (5%).

In this thesis, maximum negative effect on the survival rate after oryzalin treatment in *T. leontopetaloides* was recorded for treatment 30 µM after 48 hours where 45% of plants did not survive. Similar results of the reduction in the survival rate at higher concentration of oryzalin for a longer exposure time has also been reported in *Ullucus tuberosus* (Viehmannová et al. 2012). Zahumenická et al. (2018) also reported 0% survival at oryzalin concentration of 15 µM for 12 weeks exposure time in *Anemone sylvestris* .

Different treatment methods of oryzalin application for *in vitro* polyploidization have been reported by researchers. In this study, shoots where subcultured on fully concentrated MS medium for two days and overlaid with the oryzalin solution containing 2% DMSO. Similar treatment method was used by Viehmannová et al. (2012) for the induction of polyploidy in *Ullucus tuberosus*. Bouvier et al. (2002), however, reported the use of nutrient solution containing 7.5%

DMSO for haploid polyploidization of pear. Denaeghel et al. (2018) reported the use of liquid MS medium with the addition of oryzalin for the induction of polyploidy in *Escallonia rubra*, *Escallonia rosea*, and *Escallonia illinita*

Although the ploidy level of several tuberous plant species such as *Solanum tuberosum* L (Chauvin et al. 2003), *Manihot esculenta* Crantz (Awoleye et al. 1994) and *Smallanthus sonchifolius* (Viehmannova´ et al. 2009) have been altered using oryzalin, polyploid induction using colchicine has also been proposed for different tuberous crop species. Heping et al. (2008) also reported a polyploidy rate as high as 36.7% in *D. zingiberensis* calli immersed in 0.3% colchicine solution for 16 h prior to culture. Babil et al. (2016) reported a high rate of somaclonal polyploid variation was successfully achieved by in vitro colchicine treatment of *D. rotundata* and *D. cayenensis* where the highest rate of polyploid induction appeared after 0.1% colchicine treatment.

5.2. Evaluation of tetraploids

Polyploidy has become a trait in plant breeding yearned by most plant breeders because it is associated with altered morphology, enhanced vigor, higher pest and diseases tolerance and hybrid fertility restoring (Stebbins 1971). Morphological consequences as a results of polyploidization may include increased width/length ratio of leaves, thicker leaves and stems and more compact growth habit; however, their appearance is influence by heterozygosity, gene interactions, gene dose effects, and epigenetic phenomena (Leitch and Bennett 1997). In this study, similar morphological changes of in vitro plants such as increased leaf size, width/length ratio, changes in leaf shape, dark green colouration of leaves, thick shoots, increased in the number of roots as well as thicker roots were observed in tetraploid plants as a possibility of primary polyploidy detection.

In this research, *T. leontopetaloides* tetraploid variants tend to have rounder leaves with an obtuse shape as compared to diploid with palmately lobed leaf shape. Babil et al. (2010) also recognize similar variation in leaf shape between diploid and tetraploid variant of water yam accessions collected in Myanmar. The leaf index (width/length ratio) in tetraploid plants was higher (1.55 ± 0.12) than in diploid plants (1.37 ± 0.06) meaning that the leaves of tetraploid plants are wider as compared to the diploid plants. This results is similar to reports in *Phlox subulata* . (Zhang et al.

2008), *Paulownia tomentosa* (Tang et al. 2010), *Rosa rugosa*. (Allum et al. 2007), *Centella asiatica* L. (Kaensaksiri et al. 2011) and *Gerbera jamesonii* Bolus cv. Sciella (Gantait et al. 2011) indicating that tetraploidization usually influences leaf width more than length. On the contrary, Zahumenická et al. (2018) in *Anemone sylvestris* L, Rêgo et al. (2011) in *Passiflora edulis*, and Pansuksan et al. (2014) in one tetraploid line of *Mitracarpus hirtus* reported an enhanced leaf length in comparison with the width of leaf.

Tetraploid plants were observed to have darker green colouration leaves compared to diploids. These morphological changes may be caused by doubling in the chromosome number of the tetraploid plants and could be useful in polyploidy detection and increasing the photosynthetic activity in *T. leontopetaloides* tetraploid plants. Jadrná et al. (2010) reported similar changes in leaf colouration in colchiploids from colchicine-treated *Pelargonium* × *hortorum* .

In this study, morphological evaluation showed no significant difference in plant height between *T. leontopetaloides* diploid and the tetraploid variant. The number of shoots produced were significantly larger in diploids than in tetraploids and this also indicates a higher number of leaves in diploid. This is contrary to the report by Denaeghel et al. (2018) which indicated that tetraploid plants of *Escallonia rubra*, *Escallonia rosea*, and *Escallonia illinita* are fast growing than their diploid counterparts, however the observation of thicker and larger shoots for tetraploid plants is similar for the observation made for this research. There was also similar observation of thicker stems in polyploid in *Ullucus tuberosus* (Viehmannová et al.2012). Tetraploid genotype produced significantly higher number of roots and visual observation showed thicker and robust root development in tetraploid compared to diploid ancestral plants. This was also observed in *Escallonia rubra*, *Escallonia rosea*, and *Escallonia illinita* (Denaeghel et al. 2018)

In this study, for *in vitro* morphological evaluation of tetraploid plants, MS medium without PGRs was used. This was done to ensure the morphology of the diploid and tetraploid plants is not altered by the effect of growth hormones because plant growth hormones have been cited to have an effect on the levels of physiological stresses and somaclonal variation (Karp 1995).

In this study, tetraploid plants showed a higher survival rate (80%) in comparison to the diploid plants (60%) in the field conditions. This primary results indicates that *T. leontopetaloides* tetraploid plants have better adaptation to field condition. Examination of the sequential

chromosomal flow over a passage of time in 2x and 4x callus cultures by Lavania & Srivastava (1990) shown that the diploid cells resort to tetraploidy to overcome the cultural stress whereas the tetraploids try to maintain themselves. Levin (1983) also reported that, polyploid in flowering plants have higher tolerance to mineral and nutrient stress.

A primary evaluation of harvested microtubers in this study proved that tetraploid showed increased in desirable traits such as increased in number of tubers per plant, increased in tubers weight as well as the size of tubers. This is similar to the results obtained in *Manihot esculenta* Crantz (Awoleye 1994) indicating an increase in the size and weight of *Manihot esculenta* tubers. However, the results obtained in this study is contrary to the reported in *Ullucus tuberosus* (Viehmannová et al.2012) indicating a comparable number of tubers per plant for diploid and polyploid and significantly higher weight of tubers in diploid compared to polyploid genotype .

Change in ploidy level may result in change in the chemical composition in plants. Significant higher levels of saccharides were reported in *Smallanthus sonchifolius* (Viehmannová et al. 2009). Increased in vitamin C level and lower starch contents in *Ullucus tuberosus* octoploids compared to their diploid counterparts was also reported by Viehmannová et al. (2012). In this study chemical composition was not evaluated, hence research is needed to determine the effect of the chemical composition in *Tacca leontopetaloides*.

6. Conclusion and recommendation

6.1. Conclusion

This study provides the first report of *in vitro* induction of polyploidy in *Tacca leontopetaloides*. Tetraploids were successfully induced in all treatment concentrations (25 µM or 30 µM) and exposure time (24 or 48 hours). The highest polyploidization efficiency of 10% was achieved with 25 µM oryzalin regardless of time duration and with 30 µM for 24 hours exposure time. Survival rate 90% coupled with 10% polyploidization efficiency for oryzalin concentration of 25 µM at 24 hours exposure time make this the ideal treatment for polyploidy induction in *T. leontopetaloides* using oryzalin. Tetraploid showed altered morphological characters such increased leaf width/length ratio, rounder leaf shape and dark green colouration as an indication for polyploidy detection. MS salts fortified with 0.05 mg l^{-1} NAA in combination with 0.1 mg l^{-1} zeatin increased rooting efficiency and shoot formation in tetraploid making it the ideal medium for preparation of *T. leontopetaloides* tetraploid for *ex vitro* transfer. Tetraploid plants showed normal growth and increased survival rate under field condition in comparison to diploid plants. Tetraploid showed increased in desirable traits such as number of tubers per plant, tuber weight and tuber size.

On the basis of the preliminary results obtained in this study, tetraploid plants may be used as a material with modified morphology, growth and yield characteristics for further breeding and the generation of novel varieties of *T. leontopetaloides*.

6.2. Recommendation

This is a preliminary study and for further research purposes, it is recommended to assess field cultivation of *T. leontopetaloides* for at least eight months as suggested by literatures for this species. The detailed determination of the effect of polyploidy on chemical composition in *T. leontopetaloides* is also recommended for further research.

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