INDUCED POLYPLOIDY AS A TOOL FOR THE DEVELOPMENT OF NOVEL SOUTH AFRICAN INDIGENOUS CROPS

by

Karin F. Hannweg

Submitted in fulfillment of the academic requirements of

Doctor of Philosophy

in the Discipline of Horticultural Science

School of Agricultural, Earth and Environmental Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

South Africa

ABSTRACT

Polyploidy is a naturally-occurring phenomenon in plants and has been reported as an important pathway for evolution and speciation; it is estimated that a large percentage of flowering plants are polyploid in origin. Although the first plant polyploid was discovered over a century ago, the genetic and evolutionary implications of polyploidy have not been fully elucidated. On a more practical level, there are many opportunities for utilizing induced polyploidy as a valuable tool in traditional plant breeding programmes. South Africa has the highest recorded plant species density in the world, however, many of these species have only marginal potential due to size and other constraints. Induced polyploids may be expected to exhibit one, or more, of the following characteristics resulting in the improvement or the development of new economically important plants: larger tuber, rhizome or root size; increased flower or fruit size; enhanced flower colour intensity, improved drought tolerance, increased bio-mass; improved photosynthetic capacity; larger and/or thicker leaves; dwarfism; increased secondary metabolite production, e.g. medicinal compounds. Several plant species (*Crocosmia aurea*, *Tetradenia riparia, Siphonochilus aethiopicus* and *Plectranthus esculentus*) were selected for the induction of polyploidy and various horticultural characteristics evaluated. Methods for the successful induction of polyploidy were developed for all selected species. By evaluating various horticultural characteristics of the induced polyploids it was determined that flower size, plant vigour and nematode resistance, as well as essential oil content and bioactivity could be significantly improved in all tested species. Induced polyploidy could, therefore, have a significant impact on the development of economically-viable novel crops indigenous to southern Africa.

PREFACE

The research work contained in this thesis was carried out by the candidate while based at the Agricultural Research Council's Institute for Tropical and Subtropical Crops in Nelspruit, South Africa, under the supervision of Professor Isa Bertling of the University of KwaZulu-Natal.

The contents of this work have not been submitted in any form to another University and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

_____

Professor Isa Bertling (Supervisor)

Date:

DECLARATION 1: PLAGIARISM

I, Karin F. Hannweg, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

_____

Signed: Karin F. Hannweg

Date: _____

DECLARATION 2: PUBLICATIONS AND PRESENTATIONS FROM THIS THESIS

Details of contribution to publications that form part of, and include research presented in this thesis, are listed below":

Peer-reviewed publications

1. Hannweg, K., Sippel, A. and Bertling, I. (2013) A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea.* South African Journal of Botany, 88, 367-372.

2. Hannweg, K., de Jager, K., Sippel, A. and Bertling, I. *(2015) In vitro* induction of polyploidy and its effect on selected horticultural characteristics and essential oil composition and bioactivity of the South African aromatic plant *Tetradenia riparia (Submitted and under review for publication in South African Journal of Botany)*

3. Hannweg, K., Steyn, W. and Bertling, I. *(2015) In vitro*-induced tetraploids of *Plectranthus esculentus* are nematode-tolerant and have enhanced nutritional value. Euphytica: DOI: 10.1007/s10681-015-1547-4*)*

4. Micropropagation and *in vitro* Polyploidisation of *Siphonochilus aethiopicus* (Wild Ginger) and its Effect on Selected Horticultural Characteristics *(Accepted for publication in Acta Horticulturae – Proceedings of the International Horticulture Congress 2014, Brisbane, Australia)*

Related Conference presentations

1. Hannweg, K. and Sippel, A. (2010) Use of Polyploidy in Tropical and Subtropical Plant Improvement Programmes. IHC2010, Lisbon, Portugal.
2. Hannweg, K. and Sippel, A. (2013) Induction of polyploidy in *Hibiscus sabdariffa*, an important medicinal and nutritional plant species. Combined Congress 2013, Durban, South Africa
3. Hannweg, K., Sippel, A. and Bertling, I. (2014) Micropropagation and *in vitro* induction of polyploidy and the effect on selected horticultural characteristics of the South African aromatic plant, *Tetradenia riparia*. Combined Congress 2014, Grahamstown, South Africa. Winner of Best Southern African Society for Horticultural Science PhD Paper.
4. Hannweg. K., Visser, G., Sippel, A. and Bertling, I. (2014) Micropropagation and *in vitro* Polyploidisation of *Siphonochilus aethiopicus* (Wild Ginger) and its Effect on Selected Horticultural Characteristics. e-Poster presented at International Horticulture Congress 2014, Brisbane, Australia.
5. Hannweg, K. Visser, G., Steyn, W., and Bertling, I. (2015) A comparative study of selected horticultural characteristics of induced polyploids of *Plectranthus esculentus* and their diploid progenitors. Combined Congress 2015, George, South Africa.

Signature: _____

Date: _____

ACKNOWLEDGEMENTS

I would like to take this opportunity in thanking the Agricultural Research Council of South Africa, not only the support for the research carried out towards this thesis but also for supporting the professional development of their staff. Professor John Bower, is thanked for his initial inputs, support and academic expertise during his period as supervisor. Professor Isa Bertling, who took over the reins from Professor John Bower, is especially thanked for her support and persistence in getting things done. To my colleagues and friends, their support through thick and thin is greatly appreciated. Last but not least, a special thanks to my husband, Mark Penter, who understood all the trials and tribulations, proofread material and supported me throughout.

LIST OF CONTENTS

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Rationale for the research

South Africa has the highest plant species diversity in the world. Many of these species are not economically-viable in terms of cultivation potential due to various marginal horticultural constraints, depending on the species in question. Only a few species, albeit highly profitable, have been improved, and then only outside South Africa's borders, using conventional breeding and improvement methods. Various *Gerbera* species and *Gladiolus* spp. are key examples and have earned the developer countries (Holland, Germany) enormous revenue. With over 22 000 indigenous South African plant species, there is enormous potential for improvement. It is with this potential in mind that investigations into the development of several species with both, marginal and multiple horticultural uses was conducted to determine whether induced polyploidy (chromosome doubling), could be used to improve under-utilised species with commercial potential. There are, in fact, very few published reports on the induction and consequences of induced polyploidy in South African plant species (see Chapter 2, Section 2.6).

Chromosome doubling, or induced polyploidy, is a several decades-old technique which has been applied to plant species from a wide range of plant families (Soltis et al., 2004). Although most plant species are diploid, several species are natural polyploids with three or more sets of chromosomes. Polyploidisation is a naturally-occurring phenomenon and is widely recognised as a major mechanism of adaptation and speciation in plants and has been reviewed by a number of authors (Ramsey and Schmeske, 1998; Otto and Whitton, 2000; Adams and Wendel, 2005; Otto, 2007; Parisod, *et al*., 2010; Storme and Mason, 2014). Although exceptions occur, previous research in agricultural systems has indicated that polyploids commonly have altered physiological and phenotypical attributes not present in their progenitors. As such, these alterations have the potential to result in polyploid varieties having improved characteristics, such as larger tuber, rhizome or root size, increased or enhanced flower and/ or fruit size and/or colour intensity (Yamaguchi, 1989; Takamura and Miyajima, 1996; Gu *et al*., 2005; Notsuka *et al*., 2005; Allum *et al.*, 2007), improved drought tolerance (Riddle *et al*., 2006; Xiong *et al*., 2006; Li *et al*., 2009), increased bio-mass (Dewey, 1980), improved photosynthetic capacity, larger and/or thicker leaves (Wu amd Mooney, 2002; Eeckhout *et al*., 2004; Li *et al*., 2009), dwarfism and increased secondary metabolite production (e.g. medicinal compounds) (Gao *et al*., 1996; Berkov and Philipov, 2002).

1.2 Objectives of the study

The objectives of the study were to determine the effect and possible impact of induced polyploidy on several selected indigenous South African plant species. The species investigated in this study were chosen to explore a wide diversity of characteristics important in horticultural/agricultural industries

and, therefore, the possible impact of induced polyploidy was investigated. In each instance, however, each species has potential in its own right. Chromosome doubling and verification was achieved for four indigenous plant species and comparative studies on various wide-ranging horticultural aspects were carried out to determine the possibility of using chromosome doubling as a means of improving South African species with marginal potential.

1. *Crocosmia aurea*

   Falling stars iris, *Crocosmia aurea*, is a member of the Iridaceae family and has enormous ornamental potential. There are a number of economically-important iris species which are important cut-flower and/or ornamental crops. These include a number of *Freesia*, *Gladiolus*, *Watsonia* and *Iris* species, all of which have been improved using chromosome doubling and/or conventional breeding practices. Polyploidy usually results in larger flowers which are highly desirable on the cut flower markets or a more compact stature which is more desirable in the ornamental industry. The effect of induced polyploidy on plant and flower morphology as well as phenology was therefore investigated for *Crocosmia aurea*.

2. *Tetradenia riparia*

   Ginger Bush, *Tetradenia riparia* (Family: Lamiaceae), is an aromatic shrub that occurs throughout tropical Africa and in South Africa, has traditionally been used in the treatment of cough, dropsy, diarrhoea, fever, headache, malaria, and toothache. The essential oils are also used in the perfume industry (Hutchings, 1996; van Wyk and Gericke, 2000). Polyploidy has been reported to effect an alteration in not only the biochemical profile of the essential oils of induced polyploids, but also the volume of essential oil produced by the polyploid (Lavania, 1988; McArthur and Sanderson, 1999; Parida and Misra, 2015). As a result the relationship between the plant and pollinators, herbivores and micro-organisms could be altered. It was with this premise in mind that a study on the essential oil was carried out to determine biochemical changes as well as bioactivity against known postharvest fungal pathogens, *Geotrichium candidum* and *Penicillium digitatum*.

3. *Plectranthus esculentus*

   Plectranthus esculentus, Livingstone potato (Family: Lamiaceae), is an edible tuberous vegetable which originated in Africa. Although the tubers are edible, limited crop improvement has been achieved in this under-utilised crop with enormous potential. Further, the crop is highly susceptible to rootknot nematode, *Meloidogyne* spp., which globally causes extensive losses to a wide range of crops. Since induced polyploidy has been reported to influence tuber size and yield in other tuberous crops (Smith *et al*., 2004; Sakhanokho *et al*.; Kun-Hua *et al*., 2011), a study into the effect of polyploidy on tuber yield and nutritional value was initiated.

Further, polyploidy can also have an effect on the relationship between the induced polyploid and biotic stressors and to this end, the effect of induced polyploidy and its effect on rootknot nematode development was investigated.

4. *Siphonochilus aethiopicus*

Siphonochilus aethiopicus (wild ginger; family: Zingiberaceae) is one of only several thousand plant species used in traditional medicinal preparations in South Africa. The plant is threatened with extinction and is already extinct in the wild in the KwaZulu-Natal Province. The species is increasingly threatened in the Mpumalanga Province where small populations are reported to exist. The cone-shaped rhizomes and fleshy roots are extremely popular and are widely used in traditional medicines which include treatments for asthma, hysteria, colds, coughs and flu, as well as malaria, amongst others (Hutchings, 1996; van Wyk and Gericke, 2000). Previous reports on induced polyploids in culinary ginger, *Zingiber officinale* (Smith *et al*., 2004; Kun-Hua *et al*., 2011), showed that rhizome size and yield could be substantially improved and thus resulted in the study on induced polyploidy in *S. aethiopicus*.

All of these relationships were studied on a case-by-case basis in order to assess the polyploidisation effect on the potential of developing formal crop cultivation in the agricultural/horticultural sectors.

This thesis is presented in the form of chapters represented by manuscripts either accepted, published, or under review, in peer-reviewed journals. The Appendix contains the pdf documents of published manuscripts.

REFERENCES

Adams, K. and Wendel, J. (2005). Novel patterns of gene expression in polyploid plants. Trends in Genetics, 21: 539-543.

Allum, J.F., Bringloe, D.H. and Roberts, A.V. (2007). Chromosome doubling in *Rosa rugosa* Thunb. hybrid by exposure of *in vitro* nodes to oryzalin: The effects of node length, oryzalin concentration and exposure time. Plant Cell Reports 26, 1977–1984.

Berkov, S. and Philipov, S. (2002). Alkaloid production in diploid and autotetraploid plants of *Datura stramonium*. Pharma Biolgica, 40: 617–621.

Dewey, D.R. (1980). Some application and misapplication of induced polyploidy to plant breeding. In: Lewis WH (ed) Polyploidy: biological relevance, vol 13. Plenum Press, NewYork, pp 445–470.

Eeckhaut, T., Werbrouck, S., Leus, L., Van Bockstaele, E., Debergh, P. (2004). Chemically-induced polyploidization in *Spathiphyllum wallisii* Regel through somatic embryogenesis. Plant Cell Tissue Organ Culture, 78:241–246.

Gao, S.L., Zhu, D.N., Cai, Z.H., Xu, D.R. (1996). Autotetraploid plants from colchicine treated bud culture of *Salvia miltiorrhiza* Bge. Plant Cell Tissue and Organ Culture, 47: 73–77.

Gu, X.F., Yang, A.F., Meng, H. and Zhang, J.R. (2005). *In vitro* induction of tetraploid plants from diploid *Zizyphus jujuba* Mill. Cv. Zhanhua. Plant Cell Reports, 24:671–676

Hutchings, A. (1996). Zulu medicinal plants: an inventory. University of Natal Press, Pietermaritzburg, South Africa.

Kun-Hua, W., Jian-Hua, M., He-Ping, H. and Shan-Lin, G. (2011). Generation of autoteraploid plants of ginger (*Zingiber officinale* Rosc.) and its quality evaluation. Pharmacognasy Magazine. 7: 200-206.

Lavania, U. (1988). Enhanced productivity of the essential oil in the artificial autopolyploid of vetiver (*Vetiveria zizanioides* L. Nash). Euphytica 38, 271-276.

Li, W.D., Biswas, D.K., Xu, H., Xu, C.Q., Wang, X.Z., Liu, J.K. and Jiang, G.M. (2009). Photosynthetic responses to chromosome doubling in relation to leaf anatomy in *Lonicera japonica* subjected to water stress. Functional Plant Biology, 36:783–792.

McArthur, E.D. and Sanderson, S.C. (1999) Cytogeography and chromosome evolution of subgenus Tridentatae of *Artemisia* (Asteraceae). American Journal of Botany, 86:1754-1775.

Notsuka, K., Tsuru, T. and Shiraishi, M. (2000). Induced polyploid grapes via *in vitro* chromosome doubling. Journal of the Japanese Society for Horticultural Science, 69:543–551.

Otto, S. and Whitton, J. (2000) Polyploid incidence and evolution. Annual Review of Genetics, 34: 401-437.

Otto, S.P. (2007) The evolutionary consequences of polyploidy. Cell. 131:452–462

Parida, B. and Misra, B. (2015) Is a plant's ploidy status reflected in its metabolome? Journal of Post Doctoral Research, 3: 1-11.

Parisod, C., Holderegger, R. and Brochmann, C. (2010) Evolutionary consequences of autopolyploidy. New Phytologist, 186: 5-17.

Ramsey, J. and Schmeske, D. (1998) Pathways, mechanisms and rate of polyploid formation in flowering plants. Annual Review of Ecological Systems. 29: 467 – 501.

Riddle, N.C., Kato, A. and Birchler, J.A. (2006). Genetic variation for the response to ploidy change in *Zea mays* L. Theoretical and Applied Genetics, 114:101–111.

Sakhanokho, H., Rajasekaran, K., Kelley, R. and Islam-Faridi, N. (2009). Induced Polyploidy in Diploid Ornamental Ginger (*Hedychium muluense*) Using Colchicine and Oryzalin. HortScience, 44: 1809-1814.

Smith, M., Hamill, S., Gogel, B. and Severn-Ellis A (2004) Ginger (*Zingiber officinale)* autotetraploids with improved processing quality produced by an *in vitro* colchicine treatment. Australian Journal of Experimental Agriculture. 44: 1065–1072.

Soltis, D., Soltis, P. and Tate, J. (2004) Advances in the study of polyploidy since plant speciation. New Phytologist. 161: 173 -191.

Storme, N. and Mason, A. (2014) Plant speciation through chromosome instability and ploidy change: Cellular mechanisms, molecular factors and evolutionary relevance. Current Plant Biology, 1: 10-33.

Takamura, T., Lim, K.B. and van Tuyl, J.M. (2002). Effect of a new compound on the mitotic polyploidisation of *Lilium longiflorum* and Oriental hybrid lilies. Acta Horticulturae, 572: 37–40.

Van Wyk, B-E. and Gericke, N. (2000) People's Plants: a guide to useful plants of southern Africa. (351 pages). Briza Publications, Pretoria.

Wu, J.H. and Mooney, P. (2002). Autotetraploid tangor plant regeneration from *in vitro* citrus somatic embryogenic callus treated with colchicine. Plant Cell Tissue and Organ Culture 70: 99–104.

Xiong, Y.C., Li, F.M. and Zhang, T. (2006). Performance of wheat crops with different chromosome ploidy: root-sourced signals, drought tolerance, and yield performance. Planta, 224:710–718.

Yamaguchi M (1989). Basic studies on the flower color breeding of carnations (*Dianthus caryophyllus* L.). Bulletin of the Faculty of Horticulture, Miamikyusyu University, 19:1–79.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Polyploidisation, i.e., whole genome duplication, is a powerful strategy for the development and/or improvement of plant genotypes (Soltis *et al.*, 2004). While polyploidisation is a naturally-occurring phenomenon, it has been used for crop improvement across a wide range of plant species (Renny-Byfield and Wendel, 2014). *In vitro*-induced polyploidy induction is facilitated by the relatively small piece of tissue selected compared with methods carried out *ex vitro*; however, the conditions for polyploid induction are not only tissue-specific but also species-specific. Induced polyploidy is usually carried out by treating the target tissue with an antimitotic agent, the concentration and timing of which needs to be optimised. Following treatment with the antimitotic agent, the resulting population must be verified for polyploid production as well as mixoploidy to prevent the selection of unstable genotypes. Various verification methods such as chromosome counting and morphological observations have been used to confirm the ploidy level of treated tissues, with flow cytometry being the most commonly used method in recent times due to its efficiency and accuracy.

Induced polyploidy has several consequences for crop development and improvement; however, the effects of induced polyploidy are not predictable and are largely dependent on the species investigated. While new genetic material is not introduced into the genome in question, additional gene copies are added resulting in gene duplications and, therefore, potential changes in gene expression with subsequent changes in various horticultural characteristics Osborn *et al.*, 2003; Otto, 2007; Parisod *et al.*, 2010). These alterations may include anatomical and morphological changes, genetic adaptability and tolerance to abiotic and biotic stresses many of which have important applications, and implications for the development of superior genotypes (te Beest *et al.*, 2012).

As indicated earlier in this Chapter, very few induced polyploidy studies have been carried out on South African indigenous plant species with the aim of developing superior polyploid genotypes. As such, this study sought to apply the technique across a range of plant families and species targeting different potential uses. The studies aimed to investigate the impact of induced polyploidy, if any, on horticultural characteristics such as ornamental value, nutritional value, pest resistance/tolerance and bioactivity on selected indigenous South African plant species.

This Literature Review seeks to provide an appraisal of polyploidy and its potential for the development of improved genotypes. It includes background to methods for induction and verification of induced polyploids as well as the consequences of polyploidy in terms of various aspects of plant performance including morphological, physiological, stress tolerance and secondary metabolite production and the possible applications of these consequences on plant improvement.

2.2 Polyploidy in the Angiosperms

Polyploidy, defined as the possession of three or more complete sets of chromosomes, is acknowledged as a major mechanism of adaptation and speciation in plants (Ramsey and Schmeske, 1998; Jiao *et al*., 2011). It is estimated that at least half of all Angiosperms are polyploid and that polyploid evolution is an ongoing process (Grant, 1981; Masterson, 1994; Fawcett and Van de Peer, 2010). Extensive research on agricultural crops as well as non-agricultural crops over the last nine decades has revealed that polyploids have altered morphological and physiological characteristics when compared with their diploid progenitors. It is these alterations that have potential and have resulted in a number of studies focusing on crop/plant improvement. Although the technique is generally well-established, there has been a re-stimulation of research on polyploidy over the last ten to fifteen years (reviews by Leitch *et al*., 2004 and Soltis *et al*., 2004; Udall and Wendel, 2006; Soltis and Soltis, 2009; Tayale and Parisod, 2013). These studies have included mechanisms of polyploid formation and establishment, frequency of polyploidisation, physiological and ecological effects as well as genetic and genomic consequences of polyploidisation.

Two types of polyploids have been described, namely "autopolyploids" and "allopolyploids". Autopolyploids arise within populations of individual species as a result of duplication of a single genome (sometimes termed whole genome doubling). Allopolyploids are the product of interspecific hybridisation, *i.e*., a combination of two or more differentiated genomes (Kihara and Ono, 1926; Stebbins, 1947; Grant, 1981). Wendel and Doyle (2005) described several routes to polyploid formation; diploids may double their chromosome complement (a strict autopolyploid), while hybridisation between individuals from different species (strict allopolyploids) can also occur. While Ramsey and Schmeske's (1998) review highlighted several pathways of natural polyploid formation, with the advances in the genomics era, there is now an even greater awareness of the importance and extent of chromosome doubling in plants. Chromosome doubling events have a fundamental significance in plant speciation and adaptation and several reviews have detailed the mechanisms, frequency and potential ecological and functional consequences of polyploidisation as well as the diversity of genetic mechanisms characteristic in the evolution of genome doubling (Ramsey and Schmeske, 1998; Soltis *et al*., 2004; Wendel and Doyle, 2005). These reports suggest that there is evidence that both autopolyploids and allopolyploids are common in nature although allopolyploidy is more common. Furthermore, Hilu (1993) also reported that both types of polyploids are common in important food crops (allopolyploids include wheat, cotton and canola while autoployploids include bananas, apples and potato) thus highlighting its potential value in plant/crop improvement programmes.

The morphological, physiological and ecological consequences of polyploidy in plants, while immense, are poorly understood. Research indicates that in nature, and in agricultural systems, polyploids often

possess physiological characteristics not present in their diploid progenitors, and it is for these reasons that induced chromsosome doubling warrants investigation as a mechanism for the improvement of plant species.

2.3 *In vitro* methods of induced chromosome doubling for plant/crop improvement

Induced chromosome doubling (polyploid induction) is a powerful method for the production of transformed genotypes for breeding and improvement of plants. Polyploids are induced through two different mechanisms, each with huge potential in plant breeding. Mitotic polyploidisation involves the doubling of chromosomes in somatic tissue, whereas meiotic polyploidisation is based on the doubling of gamete chromosome number, *i.e.*, the generation of di-haploids (Ramsey and Schmeske, 1998). These 2n gametes can be used directly in direct crossings and can, therefore, reduce the breeding process by one generation. Furthermore, 2n gametes enhance progeny genetic variation resulting in higher diversity and, therefore, a potentially greater degree of expression of traits.

There are a large number of vegetatively-propagated flower and fruit species as well as agricultural crops which are natural polyploids; however, since natural polyploidy does not exist across all genera in angiosperms and chromosome doubling has potentially highly beneficial results, polyploids of economically-important crops have been artificially induced over several decades. First experiments were carried out by Blakeslee and Avery (1937) in the 1930s. These early induced polyploids were developed using plants established in the field. Colchicine was the mitotic inhibitor of choice and axillary buds were soaked in colchicine solutions (Pei, 1985). Blakeslee (1939) reported on the chromosome doubling of several plant species and for agricultural crops, *in vivo* polyploidisation was successful for sugar beet, rye and clover (Dewey, 1980). However, despite these developments, the rate of polyploidisation was extremely low with a concomitant excessively high incidence of mixoploids. These mixoploids (also known as chimeras) contain any ratio of diploid: chromosome-doubled cells randomly distributed throughout the resulting plants population making the genotype highly unstable and commercially non-viable.

In the 1960s, Murashige and Nakano (1966) reported on the first *in vitro* polyploidisation experiment using tobacco. Micropropagation provides a controlled environment in which chromosome doubling treatments can take place and has a greater potential for improving the efficiency of polyploidisation events. Furthermore, due to the nature of *in vitro* systems, pure polyploids can be harnessed from mixoploids (De Schepper *et al*., 2004; Chen and Gao, 2007; Aleza *et al*., 2009); this cannot be achieved *in vivo* where vegetative propagules are far larger than *in vitro* and propagules are restricted to large multi-cellular structures. *In vitro* chromosome doubling was regularly used as *in vitro* propagation methods evolved and has now become the method of choice for many plant species.

Chromosome doubling is usually achieved with the application of anti-mitotic agents which interfere with the plant's cell cycle (Dhooghe *et al*., 2011). Although there has been extensive research on the effects of various chemicals on polyploid induction, it is only the so-called "Group 1" chemicals which are antimitotic agents able to disrupt spindle formation and function (Vaughn, 2000). During somatic cell division (mitosis), cells have a doubled chromosome complement between the S-phase (DNA synthesis) and completion of mitosis. The cell cycle can be disrupted at metaphase with chemicals known as metaphase inhibitors. During metaphase, chemicals which interfere with the organisation of the spindle threads, or microtubules by associating with the alpha- and beta-dimers of the spindle, will prevent the migration of chromosomes during anaphase. As a result, the chromosomes do not separate resulting in cells with double the number of chromosomes (Planchais *et al.*, 2000).

Colchicine is the most commonly used antimitotic agent. It is extracted from the seeds and bulbs of *Colchicum autumnale* (Eigsti and Dustin, 1955) and is commonly used as an anti-inflammatory medication for gout. Blakeslee and Avery (1937) made use of colchicine in their early experiments on polyploid induction in the field; the chemical was also used in subsequent *in vitro* studies on polyploidisation. One of its major advantages is that it is stable under high temperatures and can, therefore, be readily sterilised with culture media without losing its efficacy (Zhang *et al.*, 2007), making polyploidy induction experiments simpler. Colchicine, however, causes sterility, abnormal growth, loss and/or rearrangement of chromosomes and is highly toxic to animal (and therefore human) cells due to its higher affinity for animal cell microtubules compared with plant microtubules; therefore, alternatives to colchicine have been sought after. Extensive research revealed that a number of herbicides on the market belonging to diverse classes of chemicals, affect mitosis as their mode of action. Chemical classes include: dinitroanilines (oryzalin and trifluralin) (Morejohn *et al.*, 1987; Verhoeven *et al*., 1990; van Tuyl *et al*., 1992; Hansen and Andersen, 1996), phosphorothioamidates (amiprophos-methyl), benzamides (pronamide), carbamates (chlorpropham, isopropyl N-(3-chlorophenyl) carbamate) and others (Molin and Khan, 1997; Vaughn, 2000). As these herbicides and pesticides also have a higher affinity for plant tubulin dimers than colchicine and other antimitotic agents, they can be used at lower concentrations and are also less toxic than colchicine, presenting alternatives to colchicine. Despite the variety of antimitotic chemicals available, the majority of publications describes the use of colchicine or oryzalin as antimitotic agents for use in chromosome doubling experiments.

Chromosome doubling *in vitro* is a multi-step process: treatment of the tissue of choice with the antimitotic agent/s of choice and a growth cycle to determine polyploidisation efficiency. Once ploidy level has been determined, the selected polyploids are proliferated for further studies. Apart from tissue choice and antimitotic agent, variables such as antimitotic agent concentration, exposure time,

application and method of polyploid verification need to be taken into account for successful chromosome doubling.

Since the advent of *in vitro* chromosome doubling, several explant (*i.e*. tissue) types have been used for a wide variety of species. Dhooghe *et al*. (2011) carried out an extensive review of explant types where polyploidisation has been successful. These include shoots, buds or shoot tips, callus, somatic or zygotic embryos, seeds, seedlings, nodal segments and tuber segments. Sometimes, the suitability of several explant types needs to be investigated depending on the species under study. Several authors have reported that polyploidisation success is dependent on explant type (Kermani *et al*., 2003; Petersen *et al*., 2003), as well as genotype (de Mello e Silva *et al*., 2000; Chauvin *et al*., 2005; Stanys *et al*., 2006; Khosravi *et al*., 2008).

For each plant species, concentration and exposure time to the antimitotic agent is critical. Low colchicine concentrations (0.5mM) are generally reported to have no effect on the cell cycle, while too high a concentration (>5mM colchicine) is lethal. High concentrations of antimitotic agents can also lead to doubling of the doubled chromosomes leading to higher, but undesirable ploidy levels (Allum *et al*., 2007). The solvent used to dissolve the antimitotic agent can also be critical; solvents such as dimethylsulphoxide (DMSO) have been reported to increase permeability and therefore facilitate increased absorption of chemicals. DMSO, however, can also induce plant mortality such that the survival rate of treated explants and plants is reduced compared with dissolving the antimitotic agent in water. In instances where DMSO is lethal, other solvents such as NaOH (Dhooghe *et al*., 2009) or ethanol (Petersen *et al*., 2002) (oryzalin), acetone (Dhooghe *et al*., 2009) (trifluralin) or water (Hamill *et al*., 1992; Dhooghe *et al*., 2009) (colchicine) have been used. Wetting agents are commonly used to improve contact with the explant surface (Eeckhaut *et al*., 2002).

2.4 Methods for verification of polyploidisation induction

There are a variety of methods of application of the antimitotic agent including direct application to the apical meristem or axillary buds, soaking of seeds in liquid antimitotic agent or soaking of embryogenic or organogenic callus in liquid antimitotic agent. Antimitotic agents can also be incorporated into the culture medium and therefore integrated into the micropropagation protocol, although treatment for a short period in liquid medium followed by culture of new polyploid shoots on regeneration medium is a common method for polyploid induction. Dhooghe *et al.* (2011) carried out an extensive review of the currently used methods for *in vitro* polyploidisation across a wide range of species.

Polyploid induction can be verified using a number of techniques, including chromosome counting, flow cytometry and evaluation of anatomical and morphological traits of the so-called induced

polyploids compared with the diploid progenitors. Chromosome counts are the most complete as they determine actual chromosome number within cells, but the method is time-consuming and labour intensive as cells must be effectively stained and visualised microscopically. Apart from three dimensional effects of cells which can lead to errors in counting chromosomes, errors readily occur when counting very small chromosomes. Flow cytometry, on the other hand, while it cannot determine chromosome number of a plant where little research has been carried out in terms of chromosome number, is an extremely efficient method for determination of ploidy level (Ochatt *et al*., 2011). In essence, cell nuclei are released from cells by chopping a representative sample of a potentially induced polyploid in a buffer, staining with a fluorescent dye which binds to DNA and analysis using a flow cytometer which aligns nuclei that fluoresce under UV light. The fluorescence emitted is proportional to the amount of DNA in a cell and can be correlated with its DNA content (Dolezal and Bartos, 2005). Flow cytometry allows for the screening of large numbers of samples within a relatively short space of time and further, very small pieces of tissue from any part of the plant may be used. Moreover, flow cytometry is an extremely useful technique to screen out mixoploids from large populations as large numbers of cells can be analysed. Early screening and detection of pure polyploids saves both time and space in research facilities (Dolezal *et al*., 2007).

Morphological and anatomical observations have also been used to verify polyploid induction, however, these may be highly inaccurate (Zlesak *et al.*, 2005). Typically, characteristics used to identify induced polyploids have included increased stomatal size and reduced distribution as well as increased stomatal aperture and guard cell size. The chloroplast number of stomatal guard cells has also been used as a marker for selection of polyploid plants. Dhooghe *et al.* (2011) reviewed a number of reports on a wide range of plant species and reported larger stomata, reduced stomatal distribution and an increased chloroplast number as features in polyploids. Although typical polyploid morphological characteristics, such as thicker stems, increased width to length ratio of leaves and decreased stomatal distribution can be used as selection criteria for polyploids, verification of chromosome doubling using other methods is necessary. As an example, Zhang *et al*. (2010) reported that only 50% of *Lagerstroemia indica* plants screened initially, based on morphological characteristics, were pure polyploids when verified using flow cytometry.

2.5 Effects of polyploidy on aspects of plant performance and the role of polyploidy in crop/plant improvement – morphological, physiological and ecological consequences

Polyploid plants can be markedly different from their diploid progenitors in terms of biochemical, cellular, morphological and physiological aspects (Stebbins, 1947). Moreover, polyploid plants are used as sources of variation and new genotypes in plant improvement programmes. In general, polyploids usually have larger cells and larger, but fewer stomata resulting in plants with thicker and larger leaves,

larger flowers as well as larger fruit. Autotetraploids may be significantly larger and have larger seeds, but fertility may be compromised. Phenology may also be affected with tetraploids flowering and fruiting later in the season compared with diploids. The shoots of polyploids may also be shorter and thicker resulting in a dwarfed appearance, a trait highly desirable in fruit tree crops in order to facilitate orchard management or in ornamentals where a more compact appearance is more desirable Ascough and van Staden, 2008)). Other novel physiological characteristics which may be expressed in polyploids include drought stress tolerance as well as pest and/or disease resistance and various characteristics relating to pre- and postharvest performance – characteristics important for commercial application in the agricultural and horticultural industries. As early as the 1930s and 1940s, it was found that polyploid plants occupied different ecological niches (usually drier or higher in altitude) compared with their diploid counterparts (Müntzing, 1936; Stebbins, 1942, Clausen *et al*., 1945). As a result, it has been postulated that polyploids may therefore be more highly adapted to adverse conditions (Soltis and Soltis, 2000). As such, it has been reported that polyploids have a higher resistance to water stress. Furthermore, palatability and digestibility as well as nutrient value are increased in certain polyploid fruit species (Dewey, 1980). In some polyploid species, tetraploids were also more tolerant to heat stress than their diploid counterparts (Zhang *et al*., 2010). These altered characteristics present serious advantages in any agricultural/horticultural industry; with an increase in chromosome number, DNA content, cell enzyme activity and cell volume increase, - these advantages are, however, not expressed in all cases. And this is the challenge!

Several researchers postulated that polyploids could have certain advantages over diploids with respect to certain attributes (Dhawan and Lavania, 1996; Levin, 2002) and that these could be exploited in terms of plant/crop improvement. Although induced chromosome doubling has been successfully used across the agricultural crop spectrum, the majority of reports and therefore success has been reported in the ornamental industry. This can be attributed to the efficient *in vitro* propagation protocols which are employed when chromosome doubling is performed *in vitro*. For many agricultural crops, successful clonal propagation *in vitro* is limited, thereby limiting knowledge of *in vitro* polyploidisation in these crops, although dihaploidisation (double haploids) has been investigated in a few crops, such as wheat (Kim and Baenziger, 2005), asparagus (Dore, 1976) and other vegetables (Juhasz *et al*., 2006). Furthermore, successful induction is a compromise between toxicity and chromosome doubling efficiency and there is a marked contrast in terms of the definition of a successful event as reviewed by Dhooghe *et al.* (2011). Success rates are reported to range from 15% to 55% and may be based on the number of treated plants or survival of treated plants. Polyploidisation may even include mixoploid numbers where individuals have a mixture of diploid and tetraploid cells scattered throughout. Success can also mean that a single confirmed induced polyploid can successfully and stably be propagated *in vitro* - but successful polyploidisation should also include hardening-off to ambient conditions.

Somatic chromosome doubling results in additional copies of existing genes and consequently many genome alterations. These alterations may be in the form of loss of duplicated genes, changes in gene expression as well as epigenetic changes which control gene expression. These effects were extensively reviewed by Osborn *et al*. (2003); Otto (2007) and Parisod *et al*. (2010). Polyploids are usually, although not in all instances, superior to their diploid progenitors in terms of trait expression. These can include phenotypic changes (Stebbins, 1947; Stebbins, 1971; Levin, 2002; Soltis *et al*., 2004; Knight and Beaulieu, 2008) as well as adaptability and tolerance to biotic and abiotic stressors (Otto and Whitton, 2000; Levin, 2002; Treier *et al*., 2009). Although reasons for these differences are not fully understood, Parisod *et al*. (2010) postulated there may be a better response to environmental changes due to increased genome flexibility. Further, as reviewed by Osborn *et al*. (2003), polyploids have an increased level of gene expression and this could result in dosage-related expression.

2.5.1 Morphology

Alterations in morphology due to polyploidy have been widely reported and reviewed by Kazi *et al.* (2015). Morphological changes such as increased leaf thickness, increased width/length ratio of the leaves, darker green colour of the leaves as well as larger flowers have all been reported. Flowers may exhibit a deeper hue, may have more petals per flower than the diploids but fewer flowers on the inflorescence stem which may also be stronger than the diploid inflorescence stem. Generally, polyploidy results in a more compact growth form due to shorter internodes. Fruit size, seed set and size is reportedly larger for polyploids although fruit quality may be compromised (Ranney, 2006).

One of the major consequences of polyploidy is cell enlargement (Stebbins, 1971) - polyploids generally have larger cells than diploids. Such an enlarged cell size is correlated with altered plant morphology, resulting in polyploids being more vigorous, taller and producing larger flowers and seeds. There is, however, a limit as to the optimum nucleic acid content, i.e., chromosome number beyond which cellular metabolism cannot operate and the plant becomes less vigorous and possibly even unviable. The growth rate of polyploids can become severely reduced as a result of the reduced metabolism due to changes in cellular structure.(Müntzing, 1936; Bennett and Leitch, 2005; Gregory, 2005); however, as far back as the 1970s, it was reported that the slower metabolism facilitated by larger cell size, may in fact facilitate plant longevity. Stebbins (1971) and Levin and Wilson (1976) reported that the occurrence of polyploids was more common in perennial herbs and woody plants which have an obvious competitive advantage over species which are annual in nature.

Not only does polyploidy commonly result in an increase in flower size, but also in a possible change in shape, thus attracting a different suite of pollinators (Segraves and Thompson, 1999). Moreover, the type of flower may also be influenced by polyploidy – some species produce higher numbers of

cleistogamic flowers which in turn influence self-pollination which could facilitate seed set under adverse conditions (Bernström, 1950). Not only is flower morphology affected by polyploidy, but phenology may also be influenced. Changes in flowering phenology, prolonged flowering and later onset of flowering have all been reported (Smith, 1946; Bose and Choudhury, 1962; Garbutt and Bazzazz, 1983; Petit *et al*., 1997). These changes in phenology could contribute to the successful establishment of polyploids which are able to avoid competition for growing conditions, space and other resources (Wolkovich and Cleland, 2010; te Beest *et al*., 2012). With increasing flower size comes increased seed size which produce strong seedlings with a more rapid growth rate, although the germination rate of polyploids is often reportedly lower than of diploids (Bretagnolle *et al*., 1995). Fast-growing seedlings have obvious competitive advantages in terms of establishment (Jakobs *et al*., 2004; Stastny *et al.*, 2005).

2.5.2 Physiology

Plant morphology is a visibly obvious alteration in polyploids of many plant species, however, polyploidy can also, but not always, have a significant impact on plant physiological processes, including plant-water-relations. Not only are plant cells generally larger in polyploids, but they also have fewer, albeit larger, stomata. This means that transpiration rates are lower and, therefore, polyploids have a higher water use efficiency than diploids (Chen and Tang, 1945; Maherali *et al*., 2009). In a study carried out by Garbutt and Bazzazz (1983), polyploid *Phlox drummondii* plants showed a greater preference for drier soil conditions and several more recent studies support these findings (Lowry and Lester, 2006; Hahn *et al.,* 2012). These observations led researchers to deduce that polyploids are better adapted to drier conditions and that, furthermore, this adaptation to and success under these conditions, could be one of the underlying factors of successful establishment of invasive species (Treier *et al*., 2009; te Beest *et al*., 2012). Gaseous exchange rates and, therefore, photosynthetic capacity, as for transpiration rates mentioned above, are expected to be lower in polyploid plants than in diploids, a phenomenon reported across a wide range of species (Levin, 2002). Warner *et al.* (1987; 1989) showed that there is a positive relationship between ploidy level and photosynthetic rate per unit leaf area in *Panicum virgatum* and *Atriplex confertifolia.*, however, the number of cells per unit leaf area decreases with increasing ploidy level; so even if the photosynthetic rate per cell is higher, overall the rate per leaf may be the same, or even lower, than of diploids. In contrast, Li *et al.* (2009) reported that under water-stressed conditions, Japanese honeysuckle polyploids outperformed their diploid progenitors in terms of photosynthetic rate. These authors also found that the polyploids generally had a higher CO_2 assimilation rate per unit leaf area. These findings supported the earlier work of Li *et al*. (1996) on *Betula papyrifera* and seem to indicate that polyploid plants are more resistant to water stress than diploids. Furthermore, these authors, and others, reported that polyploids recovered more quickly

than the diploids on rehydration (Pustovoitova *et al*., 1996 and Li *et al*., 2009). Tetraploids were also found to have smaller leaves, thicker epidermal and palisade layers and were highly pubescent compared with the the diploids. These results clearly indicate the enormous potential of polyploids in the light of climate change, an issue high on the agenda of many food-producing countries across the globe. Producing polyploids should therefore form an integral part of any breeding and improvement programme.

2.5.3 Abiotic stress tolerance

Drought (water stress) tolerance is not the only abiotic stressor where polyploids may outperform diploids. It is well-known that polyploidy may increase abiotic stress tolerance and may therefore be an important factor in the success of polyploids. Almost 150 years ago, reports of better performance by tetraploids of various species on nutrient-poor soils was reported (Rohweder, 1937; Noguti *et al.,* 1940) and more than half a decade later, it was revealed that tolerance to low nutrient levels and high salinity was likely due to greater nutrient uptake efficiency in polyploids (Meng *et al*., 2011). However, studies also showed the inverse to be true and that tolerance could also be species-specific (Cacco *et al*., 1976). Cold tolerance in polyploids is also reported to be species-specific with some authors reporting increased tolerance to low temperature (Lachmuth *et al*., 2010; Liu *et al*., 2011) while others reported high sensitivity to low temperature (Wit, 1958; Tyler *et al*., 1978). There was speculation that polyploids were able to colonise higher altitudes, however, there is little evidence for this. In contrast, there appears to be a strong correlation of polyploidy with latitude, but Soltis *et al*. (2004) reported that this correlation was not due to cold tolerance but due to the colonising ability of perennial polyploids which occur more frequently in northern latitudes. A further postulation is successful polyploid colonisation after ice age events (Brochmann *et al*., 2004). As such, greater ecological tolerance by polyploids compared with diploids remains a question and it is speculated that polyploids with greater stress tolerance may be able to establish in a wider range of habitats (Lowry and Lester, 2006) and have a wider distribution. It is possible that distribution differentiation along ecological gradients between polyploids and diploids occurs given the effect of polyploidy on growth, morphology, physiology etc. as well as higher competitive and colonisation ability.

2.5.4 Biotic stress tolerance

The interaction between polyploids and biotic stressors such as insects, soil organisms and pathogens has not been studied in great depth. However, it has been suggested that the impact of the interactions could potentially have enormous consequences on communities and ecologies (te Beest *et al.*, 2012). In

agriculture such interactions could play an important role in resistance/tolerance to biotic stressors. Polyploidisation can affect insect visitors, as pollinators or pests, through altered biochemical profile or even altered flower structure (Thompson *et al*., 1997; Thompson and Merg, 2008). In general, polyploids are reported to be resistant/ more tolerant to pathogens such as fungi (Oswald and Nuismer, 2007; Innes *et al.*, 2008) and nematodes (Mehta and Swaminathan, 1957; Busey *et al*., 1993). However, reports have shown that in some cases polyploids are more resistant to herbivory by insect pests (Choudhury *et al*., 1968), but for other species, polyploids were found to be highly susceptible (Nuismer and Thompson, 2001; Janz and Thompson, 2002).

2.5.5 Secondary metabolites

Polyploidisation events are not strictly the sum of the two parent genomes and the combination may bring about structural and functional changes resulting in gene maintenance, loss or addition. Of particular interest in terms of medicinal and aromatic plants, compounds of pharmaceutical interest are generally present in higher concentrations in polyploids than diploids and several studies involving the mechanism/s thereof have been carried out (Dhawan and Lavania, 1996; Zhang *et al*., 2005; Caruso *et al*., 2011). These authors reported that the artificial induction of polyploidy can lead to an increase not only in secondary metabolite production but also an improvement of pharmaceutical compound quality. Since both the biochemical profile and the concentration of secondary metabolites can differ in polyploids compared with diploids these changes can result in changes in the relationship/s between plants and their insect pests, pollinators and even soil organisms and microbes.

Due to the complex nature of polyploidisation, there is no 'hard and fast' method for every plant species, although it has been shown that antimitotic agent, concentration and exposure time need to be optimised for the species under study. Although colchicine is highly toxic, it is still the most widely used and successful chromosome doubling agent used across a wide range of genera. Other antimitotic agents have been used successfully for relatively few plant species but it is only a matter of time before new compounds which are more efficient and less harmful to the user, become more readily available. The success rate of polyploidisation is extremely variable and further in-depth studies are required to understand the mechanism of formation as well as the mechanism of mixoploid development. Polyploidy in the field of plant breeding has led to the development of new and improved varieties of a wide range of crop plants with improved morphological and physiological attributes in a far shorter time than conventional breeding methods. However, the understanding of the genetics behind these improved attributes is now receiving attention and studies on gene expression and regulation will enable breeding programmes to specifically target their efforts towards achieving the desired results, enabling breeders to manipulate gene expression of desirable traits (Aversano *et al.,* 2012; Clevenger et al., 2015).

2.6 Summary

Since the induction of polyploidy is a relatively complex process affecting not only additional gene insertions, but also gene expression, there is no single protocol which 'guarantees' that a polyploid can be developed. Further, there is also no certainty that such a polyploid will be a superior genotype compared with its diploid progenitor. Several questions remain unanswered - while colchicine remains the anti-mitotic inhibitor of choice, concentration and timing must still be optimised for each tissue type and species. Since there is limited knowledge overall, resulting in low success rates, the availability of new anti-mitotic agents could further facilitate insights into the sites of anti-mitotic agent activity as well as their permeability into tissues resulting in the optimisation of polyploidisation efficiency. Furthermore, deeper investigations into the development of *in vitro* regeneration protocols using tissues identified as polyploid in a specific part of the plant tissue would further enhance the success of polyploidisation studies where currently there is a 'hit or miss' approach.

Although there have been an enormous number of studies on the induction of polyploidy across a wide range of plant families as well as the consequences thereof, as highlighted in this Chapter, there is relatively little knowledge of the effects on genetic factors. Studies on genomic changes and gene expression and regulation provide the ideal opportunity to enable breeding programmes to carry out targeted manipulation of gene expression. Some of these studies are highlighted in Chapter 7.

As outlined earlier in this Chapter, decades of induced polyploidy studies in plants have resulted in the development of a number of improved, and economically-important, polyploid plants with superior characteristics compared with their diploid progenitors (Stebbins, 1947; Udall and Wendel, 2006; Soltis *et al.*, 2014).Characteristics such as alterations in colour, size and other important marketing characteristics in ornamentals, improved fruit size in fruit crops, increased secondary metabolite production, better adaptation (higher stress tolerance) to non-optimal conditions such as drought as well as tolerance to pests and diseases have resulted in induced polyploids becoming economically-important crops. Improvements such as these have potential for the development and improvement of South African indigenous plant species. South Africa has the world's highest plant species diversity and with approximately 22 000 species, an enormous gene pool which has not yet been harnessed – very few of South Africa's indigenous plant species have been improved apart from several bulbous species and *Gerbera* and, very few studies on induced polyploidy in South African plant species have been published (*Colophospermum mopane*, Rubuluza *et al.*, 2007; *Plectranthus* sp., Brits and Li, 2008; *Coccinia palmata* and *Lagenaria spaerica*, Ntuli and Zobolo, 2008; *Watsonia* spp., Ascough and van Staden, 2008; Thompson *et al.*, 2010). In this study, four South African plant species, each with a different use, but with potential for development, were investigated in order to determine whether substantial improvement of horticultural characteristics such as ornamental or nutritional value, bioactivity in terms of pest (nematode) and disease (fungal) resistance could be enhanced and/or

induced with the aim of using this technique for the development of other, diploid plants/crops into more productive or attractive polyploids.

REFERENCES

Aleza, P., Juárez, J., Ollitrault, P. and Navarro, L. (2009) Production of tetraploid plants of non-apomictic citrus genotypes. Plant Cell Reports 28:1837–1846.

Allum, J., Bringloe, D. and Roberts, A. (2007) Chromosome doubling in a *Rosa rugosa* Thunb. hybrid by exposure of *in vitro* nodes to oryzalin: the effects of node length, oryzalin concentration and exposure time. Plant Cell Reports, 26: 1977-1984.

Ascough, G. and van Staden, J. (2008) Effectiveness of Colchicine and Oryzalin at inducing polyploidy in *Watsonia lepida* N.E. Brown. HortScience, 43: 2248-2251.

Aversano, R., Ercolano, M., Caruso, I., Fasano, C., Rosellini, D. and Carputo, D. (2012) Molecular Tools for Exploring polyploid genomes in Plants. International Journal of Molecular Science, 13: 10316-10335.

Bennett, M.D. and Leitch, I.J. (2005) Nuclear DNA Amounts in Angiosperms: Progress, Problems and Prospects. Annals of Botany, 95 (1): 45-90.

Bernström, P. (1950) Cleisto- and chasmogamic seed setting in di- and tetraploid *Lamium amplexicaule*. Hereditas, 36: 492-506.

Blakeslee, A. (1939) The present and potential service of chemistry to plant breeding. American Journal of Botany, 26: 163-172.

Blakeslee, A. and Avery, A. (1937) Methods of inducing doubling of chromosomes in plants by treatment with colchicine. Journal of. Heredity, 28: 393-411.

Bose, R.B. and Choudhury, J.K. (1962) A comparative study of the cytotaxonomy, palynology and physiology of diploid and polyploid plants from *Ocimum kilimandscharicum* Guerke and their yield of raw material and volatile contents. Caryologia, 15: 435-453.

Bretagnolle, F., Thompson, J.D. and Lumaret, R. (1995) The influence of seed size variation on seed germination and seedling vigour in diploid and tetraploid *Dactylis glomerata*. Annals of Botany, 76: 607-615.

Brits, G.J. and Ling Li (2008). Polyploid breeding of wild South African *Plectranthus* (spurflowers) as new flowering pot plants. Acta Horticulturae, 774: 437-442.

Brochmann, C., Brysting, A.K. and Alsos, I.G. (2004). Polyploidy in arctic plants. Biological Journal of the Linnean Society, 82: 521–536.

Busey, P., Giblin-Davis, R. and Center, B. (1993) Resistance in Stenotaphrum to the sting nematode. Crop Science, 33:1066-1070.

Cacco, G., Ferrari, G., Lucci, G. (1976). Uptake efficiency of roots in plants at different ploidy levels. Journal of Agricultural Science, 87: 585–589.

Caruso, I., Lepore, L., de Tommasi, N., dal Piaz, F., Frusciante, L., Aversan, R., Garramone, R. and Carputo, D. (2011) Secondary metabolite profile in induced tetraploids of wild *Solanum commersonii* Dun. Euphytica, 8: 2226 – 2237.

Chauvin, J., Label, A. and Kermarrec, M. (2005) *In vitro* chromosome doubling in tulip (*Tulipa gesneriana* L.) Journal of Horticultural Science and Biotechnology, 80: 693-698.

Chen, L. and Gao, S. (2007) *In vitro* tetraploid induction and generation of tetraploids from mixoploids in *Astragalus membranaceus*. Scientia Horticulturae, 112: 339-344.

Chen, S.L. and Tang, P.S. (1945) Studies on colchicine-induced autotetraploid barley. III. Physiological studies. American Journal of Botany, 32:177-179.

Choudhury, J.B., Ghai, B.S., Sareen, P.K. (1968) Studies on the cytology and fertility in induced polyploids of the self-incompatible *Brassica campestris* var. brown sarson. Cytologia, 33: 269–275.

Clausen, J., Keck, D.D. and Hiesey, W.M. (1945). Experimental studies on the nature of species. 2. Plant evolution through amphiploidy and autoploidy, with examples from Madiinae. Publications of the Carnegie Institution of Washington 564: 1–174.

Clevenger, J., Chavarro, C., Pearl, S., Ozias-Akins, P. and Jackson, S. (2015) Single nucleotide polymorphism identification in polyploids: A review, example and recommendations. Molecular Plant, 8: 831-846.

De Mello e Silva, M., Callegari, J. and Zanettini, B. (2000) Induction and identification of polyploids in *Cattleya intermedia* L. (Orchidaceae) by *in vitro* techniques. Ciencia Rural, 30: 105 – 111.

De Schepper, S., Leus, L., Eeckhaut, T., Van Bockstaele, E., Debergh, P. and De Loose, M. (2004) Somatic polyploid petals: regeneration offers new roads for breeding Belgian pot azaleas. Plant Cell Tissue Organ Cult. 76:183–188.

Dewey, D. (1980) Some application and misapplication of induced polyploidy to plant breeding. In: Lewis, W. (ed) Polyploidy: biological relevance, vol. 13. Plenum Press, New York, pp 445-470.

Dhawan, O.P. and Lavania, U.C. (1996) Enhancing the productivity of secondary metabolites via induced polyploidy: a review. Euphytica, 87: 81-89.

Dhooghe, E., Denis, S., Eeckhaut, T., Reheul, D. and Van Labeke, M.C. (2009) *In vitro* induction of tetraploids in ornamental *Ranunculus*. Euphytica, 168:33–40.

Dhooghe, E. Van Laere, K., Eekhaut, T., Leus, L. and Van Huylenbroeck, J. (2011) Mitotic chromosome doubling of plant tissues *in vitro*. A review. Plant Cell Tissue and Organ Culture, 104: 359-373.

Dolezal, J. and Bartos, J. (2005) Plant DNA flow cytometry and estimation of nuclear genome size. Annals of Botany, 95: 99-110.

Dolezal, J., Greilhuber, J. and Suda, J. (Eds). (2007) Flow cytometry with Plant Cells: Analysis of Genes, Chromosomes and Genomes. Wiley-VCH, Verlag GmbH & Co. KGaA, Weinheim.

Dore, C. (1976) Chromosome doubling of asparagus (*Asparagus officinalis*) haploids by *in vitro* culture of colchicine-treated meristems. Annals Amelioration Plant 26:647–653

Eeckhaut, T., Samyn, G., and Van Bockstaele, E. (2002) *In vitro* polyploidy induction in *Rhododendron simsii* hybrids. Med. Fac. Landbouww., 66:451-454.

Eigsti, O. J. and Dustin, P. (1955). *Colchicine in Agriculture, Medicine, Biology and Chemistry,* pp. 470. Aimes, Iowa: Iowa State Coll. Press.

Fawcett, J., Van de Peer, Y. (2010) Angiosperm polyploids and their road to evolutionary success. Trends in Evolutionary Biology, 1:e3: 16-21.

Garbutt, K. and Bazzaz, F.A. (1983) Leaf demography, flower production and biomass of diploid and tetraploid populations of *Phlox drummondii* Hook. on a soil moisture gradient. New Phytologist, 93: 129-141.

Grant, V. (1981) Plant Speciation. New York: Columbia University Press. 2nd edition. 563 pages.

Gregory, T.R. (2005). The evolution of the genome (Burlington, MA: Elsevier Academic).

Hahn, M.A., van Kleunen, M., Müller-Schärer, H. (2012) Increased Phenotypic Plasticity to Climate May Have Boosted the Invasion Success of Polyploid *Centaurea stoebe*. PLoS ONE 7(11): e50284. doi:10.1371/journal.pone.0050284.

Hamill, S., Smith, M. and Dodd, W. (1992) *In vitro* induction of banana autotetraploids by colchicine treatment of micropropagated diploids. Australian Journal of Botany 40:887–896.

Hansen, N. and Andersen, S. (1996) *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin and APM in *Brassica napus* microspore culture. Euphytica, 88: 159-164.

Hilu K (1993) Polyploidy and the evolution of domesticated plants. American Journal of Botany, 80:1494-1499.

Innes, R.W., Ameline-Torregrosa, C. and Ashfield, T. (2008). Differential accumulation of retroelements and diversification of NB-LRR disease resistance genes in duplicated regions following polyploidy in the ancestor of soybean. Plant Physiology, 148: 1740–1759.

Jakobs, G., Weber, E. and Edwards, P.J. (2004) Introduced plants of the invasive *Solidago gigantea* (Asteraceae) are larger and grow denser than conspecifics in the native range. Diversity and Distributions, 10: 11-19.

Janz, N. and Thompson, J.N. (2002) Plant polyploidy and host expansion in an insect herbivore. Oecologia, 130: 570-575.

Jiao, Y., Wickett, N. and Ayyampalayam, S., et al. (2011) Ancestral polyploidy in seed plants and angiosperms. Nature, 473: 97-100.

Juhasz, A., Venczel, G., Sagi, Z., Kristof, Z., Vagi, P., Gajdos, L. and Zatyko, L. (2006) Production of doubled haploid breeding lines in case of paprika, spice paprika, eggplant, cucumber, zucchini and onion. Acta Horticulturae 725:845–853.

Kazi, N., Dawane, P. and Patil, U. (2015) Polyploidy in Ornamentals. A review paper. Journal of Global Biosciences, 4: 1768 – 1773.

Kermani, M., Sarasan, V., Roberts, A., Yokoya, K., Wentworth, J. and Sieber, V. (2003) Oryzalin-induced chromosome-doubling in Rosa and its effect on plant morphology and pollen viability. Theoretical and Applied Genetics, 107: 1195-1200.

Khosravi, P., Kermani, M., Nematzadeh, G., Bihamta, M. and Yokoya, K. (2008) Role of mitotic inhibitors and genotype on chromosome doubling of Rosa. Euphytica, 160: 267-275.

Kihara, H. and Ono, T. (1926) Chromosomenzahlen und systematische Gruppierung der Rumex-Arten. Zeitschrift für Zellforschung und Mikroskopische Anatomie. 4: 475-481.

Kim, K. and Baenziger, P. (2005) A simple wheat haploid and doubled haploid production system using anther culture. In Vitro Cell and Developmental Biology-Plant 41:22–27.

Knight C.A. and Beaulieu, J.M. (2008). Genome size scaling through phenotype space. Annals of Botany, 101: 759–766.

Lachmuth, S., Durka, W. and Schurr, F.M. (2010). The making of a rapid plant invader: genetic diversity and differentiation in the native and invaded range of *Senecio inaequidens*. Molecular Ecology, 19: 3952–3967.

Leitch, A., Soltis, D., Soltis, P., Leitch, I. and Pires, J. (2004) Biological relevance of polyploidy: ecology to genomics. Biological Journal of the Linnean Society, 82: 409-700.

Levin, D.A. (2002) The role of chromosomal change in plant evolution. Oxford University Press, New York, New York, USA. 240 pages.

Levin, D.A. and Wilson, A.C. (1976) Rates of evolution in seed plants: net increase in diversity of chromosome numbers through time. Proceedings of the National Academy of Science, USA, 73: 2086 – 2090.

Li, W.L., Berlyn, G.P. and Ashton, P.S. (1996) Polyploids and their structural and physiological characteristics relative to water deficit in *Betula papyrifera* (Betulaceae). American Journal of Botany 83, 15–20.

Li, W-D, Biswas, D.K., Hong, X., Chang-Qing, X., Wang, X-Z., Liu, J-K. and Jiang, G-M. (2009) Photosynthetic responses to chromosome doubling in relation to leaf anatomy in *Lonicera japonica* subjected to water stress. Functional Plant Biology, 36: 1–10.

Liu, S.Y., Chen, S.M., Chen, Y., Guan, Z.Y., Yin, D.M. and Chen, F.D. (2011) *In vitro* induced tetraploid of *Dendranthema nankingense* (Nakai) Tzvel. shows an improved level of abiotic stress tolerance. Scientia Horticulturae, 127: 411–419.

Lowry, E. and Lester, S.E. (2006) The biogeography of plant reproduction: potential determinants of species' range sizes. Journal of Biogeography 33: 1975–1982.

Maherali, H., Walden, A.E. and Husband, B.C. (2009) Genome duplication and the evolution of physiological responses to water stress. New Phytologist, 184: 721-731.

Masterson, J. (1994) Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science. 264: 421-24.

Mehta, R. and Swaminathan, M. (1957) Studies on induced polyploidy in forage crops. Indian Journal of Genetics and Plant Breeding, 17:27-57.

Meng, H.B., Jiang, S.S. and Hua, S.J. (2011). Comparison between a tetraploid turnip and its diploid progenitor (*Brassica rapa* L.): the adaptation to salinity stress. Agricultural Sciences in China, 10: 363–375.

Molin, W. and Khan, R. (1997) Mitotic disruptor herbicides: recent advances and opportunities. IN: Roe, R. (ed) Herbicide activity: toxicology, biochemistry and molecular biology. IOS Press, Burke, pp 143-158.

Morejohn, L., Buraeu, T., Molebajer, J., Bajer, A. and Fosket, D. (1987) Oryzalin, a dinitroanline herbicide, binds to plant tubulin and inhibits microtubule polymerisation *in vitro*. Planta, 172: 252-264.

Müntzing, A. (1936). The evolutionary significance of autopolyploidy. Hereditas (Lund), 21: 263–378.

Murashige, T, and Nakano, R. (1966) Tissue culture as a potential tool in obtaining polyploid plants. J. Heredity, 57: 114-118.

Noguti, Y., Oka, H., Otuka, T. (1940) Studies on the polyploidy of *Nicotiana* induced by the treatment with colchicine. II. Growth rate and chemical analysis of diploid and its autotetraploids in *Nicotiana rustica* and *N. tabacum*. Japanese Journal of Botany, 10: 343–364.

Ntuli, N. and Zobolo, A. (2008) Effect of water stress on growth of colchicine-induced polyploid *Coccinea palmata* and *Lagenaria sphaerica* plants. African Journal of Biotechnology, 7: 3548-3652.

Nuismer, S.L. and Thompson, J.N. (2001) Plant polyploidy and non-uniform effects on insect herbivores. Proceedings of the Royal Society B: Biological Sciences, 268:1937-1940.

Ochatt, S., Patat-Ochatt, E. and Moessner, A. (2011) Ploidy level determination within the context of *in vitro* breeding. Plant Cell Tissue and Organ Culture, 104: 329-341.

Osborn, T., Pires, J., Birchler, J., Auger, D., Chen. Z., Lee, H., Comai, L., Madlung, A., Doerge, R., Colot, V. and Martienssen, R. (2003) Understanding mechanisms of novel gene expression in polyploids. Trends in Genetics, 19: 141-147.

Oswald, B.P. and Nuismer, S.L. (2007) Neopolyploidy and pathogen resistance. Proceedings of the Royal Society B: Biological Sciences, 274: 2393-2397.

Otto, S. (2007) The evolutionary consequences of polyploidy. Cell, 131: 452-462.

Otto, S. and Whitton, J. (2000) Polyploid incidence and evolution. Annual Review of Genetics, 34: 401-437.

Parisod, C., Holderegger, R. and Brochmann, C. (2010) Evolutionary consequences of autopolyploidy. New Phytologist, 186: 5-17.

Pei, X. (1985) Polyploidy induction and breeding. Shanghai Science and Technology press, Shanghai, pp 95-98.

Petersen K.K., Hagberg, P. and Kristiansen, K. (2002) *In vitro* chromosome doubling of *Miscanthus sinensis*. Plant Breeding 121:445–450

Petersen, K., Hagberg, P. and Kristiansen, K. (2003) Colchicine and oryzalin-mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. Plant Cell Tissue and Organ Culture, 73: 137-146.

Petit, C., Lesbros, P., Xuejun, G. and Thompson, J.D. (1997) Variation in flowering phenology and selfing rate across a contact zone between diploid and tetraploid *Arrhenatherum elatius* (Poaceae). Journal of Heredity, 79: 31-40.

Planchais, S., Glab, N., Inze, D. and Bergouniux, C. (2000) Chemical inhibitors: a tool for plant cell cycle studies. FEBS Letters 476: 78-83.

Ramsey, J. and Schmeske, D. (1998) Pathways, mechanisms and rate of polyploid formation in flowering plants. Annual Review of Ecological Systems. 29: 467 – 501.

Ranney, T. (2006) Polyploidy: From Evolution to new plant Development. Combined Proceedings of the International Plant Propagator's Society, 56: 137 – 142.

Ranney, T. (1996) Polyploidy: From evolution to new plant development. Proceedings of the International Plant Propagator's Society, 56: 604-607.

Renny-Byfield, S. and Wendel, J. (2014) Doubling down on genomes: polyploidy and crop plants. American Journal of Botany, 101: 1711-1725.

Rohweder, H. (1937). Versuch zur Erfassung der mengenmässigen Bedeckung desDarß und Zingst mit polyploiden Pflanzen. Planta, 27: 501–549.

Rubuluza, T., Nikolova, R., Smith, M. and Hannweg, K. (2007) *In vitro* induction of tetraploids in *Colophospermum mopane* by colchicine. South African Journal of Botany, 72: 259-261.

Segraves, K.A. and Thompson, J.N. (1999) Plant polyploidy and pollination: floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. Evolution, 53: 1114-1127.

Smith, H.E. (1946) *Sedum pulchellum*: a physiological and morphological comparison of diploid, tetraploid and hexaploid races. Bulletin of the Torrey Botanical Club, 73: 495-541.

Soltis, P. and Soltis, D. (2009) The role of hybridisation in plant speciation. Annual Review of Plant Biology, 60: 561-588.

Soltis, D., Soltis, P. and Tate, J. (2004) Advances in the study of polyploidy since plant speciation. New Phytologist. 161: 173 -191.

Soltis, D.E., Soltis, P.S., Pires, J.C., Kovarik, A., Tate, J.A. and Mavrodiev, E. (2004). Recent and recurrent polyploidy in *Tragopogon* (Asteraceae): cytogenetic, genomic and genetic comparisons. Biological Journal of the Linnean Society, 82: 485–501.

Soltis, D. E., Visger, C.J. and Soltis, P. (2014) The polyploidy revolution then… and now: Stebbins revisited. American Journal of Botany, 101: 1057-1078.

Stanys, V., Weckman, A., Staniene, G. and Duchovskis, P. (2006) *In vitro* induction of polyploidy in Japanese quince (*Chaenomeles japonica*). Plant Cell Tissue and Organ Culture, 84: 263-268.

Stastny, M., Schaffner, U. and Elle, E. (2005) Do vigour of introduced populations and escape from specialist herbivores contribute to invasiveness? Journal of Ecology, 93: 27-37.

Stebbins, G. (1942). Polyploid complexes in relation to ecology and the history of floras. American Naturalist, 76: 36–45.

Stebbins, G. (1947) Types of polyploids: Their classification and significance. Adv. Genet. 1: 403-429.

Stebbins, G.L. (1971). Chromosomal evolution in higher plants. Addison-Wesley, London, UK. 216 pages.

Tayale, A. and Parisod, C. (2013) Natural pathways to polyploidy in plants and consequences for genome reorganisation. Cytogenetic and Genome Research, 140: 79-96.

te Beest, M., Le Roux, J.J., Richardson, D.M., Brysting, A.K., Suda, J., Kubešová, M. and Pyšek, P. (2012) The more the better? The role of polyploidy in facilitating plant invasions. Annals of Botany, 109: 19-45. Review.

Thompson, J.N. and Merg, K.F. (2008) Evolution of polyploidy and the diversification of plant–pollinator interactions. Ecology, 89: 2197-2206.

Thompson, D., Anderson, N. and van Staden, J. (2010). Colchicine-induced Somatic Polyploids from *In Vitro*-germinated Seeds of South African *Watsonia* Species. HortScience, 45: 1398-1402.

Thompson, J.N., Cunningham, B.M., Segraves, K.A., Althoff, D.M. and Wagner, D. (1997) Plant polyploidy and insect/plant interactions. American Naturalist, 150: 730-743.

Treier, U., Broennimann, O. and Normand, S. (2009) Shift in cytotype frequency and niche space in the invasive plant *Centaurea maculosa*. Ecology, 90: 1366-1377.

Tyler, B., Borrill, M., Chorlton, K. (1978). Studies in *Festuca* X. Observations on germination and seedling cold tolerance in diploid *Festuca pratensis* and tetraploid *F. pratensis* var. *apennina* in relation to altitude distribution. Journal of Applied Ecology, 15: 219–226.

Udall, J. and Wendel, J. (2006) Polyploidy and Crop Improvement. Crop Science (The Plant Genome supplement), 46: S-3-S-14.

Van Tuyl, J., Meijer, H. and Van Dien, M. (1992) The use of oryzalin as an alternative for colchicine *in vitro* doubling of *Lilium* and *Nerine*. Acta Horticulturae, 325: 625-630.

Vaughn, K. (2000) Anticytoskeletal herbicides. In: Nick, P. (ed) Plant microtubules, potential for biotechnology. Springer, Berlin, pp 193-205.

Verhoeven, H., Ramulu, K. and Dijkhuis, P. (1990) A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of *Nicotiana plumbaginfolia*. Planta: 408-414.

Warner, D.A., Ku, M.S.B. and Edwards, G.E. (1987) Photosynthesis, leaf anatomy, and cellular constituents in the polyploid C4 grass *Panicum virgatum*. Plant Physiology, 84: 461–466.

Warner, D.A. and Edwards, G.E. (1989) Effects of Polyploidy on Photosynthetic Rates, Photosynthetic Enzymes, Contents of DNA, Chlorophyll, and Sizes and Numbers of Photosynthetic Cells in the C4 Dicot *Atriplex confertifolia*. Plant Physiology, 91: 1143–1151.

Wendel, J. and Doyle, J. (2005) Polyploidy and evolution in plants. In: Henry, R. (ed) Plant diversity and evolution: Genotypic and phenotypic variation in higher plants. CAB International.

Wit, F. (1958). Tetraploid Italian grass (*Lolium multiflorum* Lam.). Euphytica, 7: 47–58.

Wolkovich, E.M. and Cleland, E.E. (2010) The phenology of plant invasions: a community ecology perspective. Frontiers in Ecology and the Environment, 9: 287-294.

Zhang, A.M., Chang, L. and Xue, J.P. (2005) Progress in research on inducing the polyploid of medicinal plants. Zhongguo Zhong Yao Za Zhi. 30(9):645-9. Review. in Chinese

Zhang, J., Zhang, M. and Deng, X. (2007) Obtaining autotetraploids *in vitro* as a high frequency in *Citrus sinensis*. Plant Cell Tissue and Organ Culture, 89: 211-216.

Zhang Q., Luo F., Liu L. and Guo, F. (2010) *In vitro* induction of tetraploids in crape myrtle (*Lagerstroemia indica* L.). Plant Cell Tissue Organ Culture, 101:41–47.

Zlesak, D., Thill, C., Anderson, N. (2005) Trifluralin-mediated polyploidisation of *Rosa chinensis* minima (Sims) Voss seedlings. Euphytica, 141:281–290.

CHAPTER 3

A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea*

K. Hannweg[a,b], A. Sippel[a], I. Bertling[b]
[a]Agricultural Research Council – Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, South Africa
Karin@arc.agric.za (corresponding author)
Phone +27 13 753 7000
Fax +27 13 752 3854
[b]Horticultural Science, School of Agricultural, Earth and Environmental Sciences, Private Bag X01, University of KwaZulu-Natal, Scottsville, 3209, South Africa

Accepted and published in South African Journal of Botany, 88 (2013) 367-372.
Impact factor: 1.34 (2013)

This manuscript is formatted according to the 2013 formatting requirements of South African Journal of Botany.

A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea*

K. Hannweg[a,b], A. Sippel[a], I. Bertling[b]

[a]Agricultural Research Council – Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, South Africa

[b]Horticultural Science, School of Agricultural, Earth and Environmental Sciences, Private Bag X01, University of KwaZulu-Natal, Scotsville, 3209, South Africa

Karin@arc.agric.za (corresponding author)

Phone +27 13 753 7000

Fax +27 13 752 3854

Key Words: chromosome doubling, *in vitro* breeding, Iridaceae, micropropagation

Abstract

South Africa is home to approximately 10% of the world's flora, many of which are endemic to the country. A large number of South African genera have been improved for horticultural use and many of these are economically important as cut flowers or ornamentals on international markets. The genus *Crocosmia*, an attractive member of the family Iridaceae, has potential both as an ornamental plant and for cut flower production, although market potential of the species may be increased by improving the size of the flowers and inflorescence. Polyploidy has been used as a tool in the improvement of ornamental plants and has led to the development of several improved ornamental species. This study established a micropropagation protocol for *C. aurea,* using seed as the source material. Tetraploidy was induced by treating seeds with colchicine. These seeds were subsequently germinated and multiplied *in vitro* using the established protocol. The resulting tetraploid plantlets were successfully hardened-off and used to study the effect of the induced tetraploidy on the plant characteristics. The tetraploid (4n) plants were found to have longer, wider leaves as well as

longer inflorescence stems and fewer, but larger, flowers than their diploid (2n) counterparts. These polyploid selections have potential in the ornamental/floriculture trade.

1. Introduction

The family Iridaceae comprises 65 genera and over 2000 species, of which 38 genera and more than half the species occur in South Africa. The plants are cormous or rhizomatous and have sword-like leaves. In general, the flowers are attractive and many species are important garden ornamentals across the globe. Although numerous species of the family have been improved by plant breeders throughout the world (Niederwieser et al., 2002; Ascough et al., 2009), there are many more species with potential for further horticultural development. *Crocosmia aurea* is no exception. This iris has bright orange-red flowers which appear from January to June on a branched inflorescence, following which a fleshy seed capsule containing purple-black seeds develops. The plant is wide-spread in the eastern parts of South Africa, where it occurs predominantly in moist areas from the coast to 2000 m above sea level (Pooley, 1993). Owing to its inherent beauty, *C. aurea* was selected for investigation of its potential as an ornamental plant and for cut flower production.

Conventional breeding has resulted in substantial improvement of genera within the family Iridaceae and many of these, such as *Gladiolus, Iris* and *Freesia* are important cut flowers on both local and international markets. The development of polyploid (chromosome doubling) induction protocols offer enormous potential for further improvement in the family. Naturally-occurring polyploidy is a phenomenon that has provided an important pathway for evolution and speciation in plants. Although the first polyploid was discovered over a century ago, the genetic and evolutionary implications of polyploidy are still being elucidated (Yang et al., 2011). The relative ease with which artificial induction of polyploidy can be achieved provides an opportunity for using this naturally-occurring phenomenon as a valuable tool in plant breeding programmes, where polyploidy has been used extensively as a tool for creating novelty in ornamental crops (Levin, 1983; Väinölä, 2000; Ascough et al., 2008). In general, tetraploids have larger flowers and fruit than their diploid counterparts and furthermore, because of their altered blooming periods, may have wider harvesting and marketing windows (Levin, 1983). These factors are particularly important in ornamental plants and cut flowers, when the potential for commercialisation is addressed. The artificial induction of polyploidy

has been reported for a number of South African iridaceous genera, including *Watsonia* (Ascough et al., 2007; Ascough et al., 2008) and *Gladiolus* (Suzuki et al., 2005).

Because naturally-occurring polyploid genotypes are usually unavailable, polyploidy is typically induced in breeding programmes through mitotic spindle inhibition or microtubule polymerization, often by exposure to colchicine (Caperta et al., 2006). Polyploid induction depends on the concentration of colchicine, the duration of exposure, explant type, and tissue penetrability (Allum et al., 2007). Colchicine-induced polyploidy is characterized by low induction rates and a high frequency of chimeras or mixoploids which must be screened out of the population; this is most commonly achieved through flow cytometry analysis (Galbraith et al., 1997). If colchicine-induced pure tetraploids are not produced, rapid *in vitro* proliferation can be used to segregate pure tetraploids from chimeras, due to the nature of the *in vitro* proliferation system.

Micropropagation has increasingly become a valuable tool for breeders, assisting in releasing new selections and cultivars into the market more rapidly. Ascough et al. (2009) reported that the first published record of *Crocosmia* micropropagation was by Koh et al. in 2007. Ovaries and florets of *C. crocosmiiflora* were cultured with 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) or kinetin. Callus formed on ovaries at low frequencies (1–28%) with BA and 2,4-D, but rooting was prolific with either NAA or 2,4-D. When florets were used as starting material, roots and corms were induced using a combination of kinetin with either 2,4-D or NAA. These corms produced shoots when kinetin was applied, callus when 2,4-D was used in combination with BA, and corms when kinetin was used in combination with 2,4-D.

The primary objective of this study was to develop a rapid and efficient protocol for the micropropagation of *C. aurea,* to establish methods for polyploid induction and to assess selected floral characteristics of the resulting polyploid plants, with a view to the development and improvement of the species.

2. Materials and methods

2.1. Development of micropropagation protocols

Diploid seeds (obtained from Silverhill Seeds, Cape Town) of *C. aurea* were used as starting material to develop a micropropagation protocol. Five hundred seeds were sterilised for 20 min using a 1% [w/v] calcium hypochlorite solution. The sterilised seeds were rinsed three times with sterile distilled water before being cultured on standard MS medium (Murashige and Skoog, 1962 [MS]) containing 30g l^{-1} sucrose, adjusted to pH 5.7 and sterilized by autoclaving for 20 min at 121 °C at 1 bar. Each seed was germinated in a glass tube containing 10 ml medium. The germinated seedlings were then transferred to MS medium containing one of four concentrations of BAP - 0.0 µM, 4.4 µM, 8.8 µM or 13.2 µM - to determine the most appropriate medium for plantlet multiplication. Each of the four BAP treatments comprised three replicates with 20 plantlets per replicate. Plantlets were maintained at 25 to 27 °C under a 16/8 h light/dark regime with cool white fluorescent light (81 µmol m^{-2} s^{-1}, Phillips 65W), and were subcultured every 4 to 6 weeks. Once multiplied and rooted, the medium was rinsed from the roots and plantlets were hardened-off for four weeks in a mistbed (housed within a polycarbonate tunnel maintained at 15 to 27 ºC) in Speedling® trays containing a 1:1 (v/v) mixture of composted pine bark and coarse river sand, before being planted out into 2 l potting bags containing a 1:1 (v/v) mixture of composted pine bark and sand. Plants were thereafter maintained in a shadehouse (40% shadecloth) at ambient conditions. Plants were watered daily on an irrigation system and Osmocote® was applied as a slow-release fertiliser.

2.2. In vitro induction of polyploidy

Diploid seeds of *C. aurea* were used as starting material to produce tetraploid plants, with the protocol described above being used to multiply plantlets generated from the treated seed. Five hundred diploid seeds were physically scarified to facilitate colchicine uptake and then treated, under aseptic conditions, with a 25 µM sterile colchicine solution for 3 days or, alternatively, a 0.25 µM solution overnight (based upon results previously obtained in our laboratory). The treated seeds were cultured on the previously determined optimum *in vitro* medium (MS medium supplemented with 4.4 µM BAP). Germination rate was recorded over six weeks, as was the final germination percentage. Proliferation rate (number of shoots per 4-6 week subculture interval), shoot height and rooting percentage were measured to determine differences between the two colchicine treatments as well as between diploid and tetraploid shoot cultures. Plants were maintained *in vitro* until they were large enough for ploidy analysis

(approximately 12 weeks after treatment) and subculture. The experiment was repeated three times.

2.3. Ploidy analysis and proliferation of confirmed tetraploid plants

The ploidy level of treated seedlings was verified using a Partec PA ploidy analyser (Partec, Germany). Samples were prepared for flow cytometry analysis using approximately 1 cm^2 of leaf tissue. The tissue was macerated with a razor blade in 125 µl of nucleus extraction solution (Partec, Germany), after which the homogenate was filtered through a 50 µm mesh filter. The isolated nuclei were stained with 1250 µl 4'-6-diamidino-2-phenylindole (DAPI) stain (Partec, Germany) prior to commencing flow cytometry analysis. Nuclei isolated from untreated, diploid plantlets were used as a standard. Histograms were analysed using the Partec software package. Seedlings confirmed as tetraploid were proliferated on MS medium containing 4.4 µM BAP under the same conditions described above. Plantlets were hardened-off as described above and used to determine polyploidy effects on various horticultural characteristics.

2.4. Morphological characterisation of tetraploids

In order to evaluate whether polyploidy induction had an effect on the horticultural characteristics of *Crocosmia*, diploid and tetraploid plantlets were proliferated and maintained as described above. The following characteristics were evaluated once the micropropagated plants had hardened-off and reached maturity: leaf width and length; flower diameter; petal width and length; stigma, stamen and anther length; inflorescence diameter and length, as well as flower bud number per inflorescence.

2.5. Statistical analysis

The experimental layout for all experiments was a complete randomised design (CRD) with 3 replications. An analysis of variance (t-test) was performed at the 5% significance level.

3. **Results and Discussion**

3.1. Development of a simple method for in vitro micropropagation of C. aurea

Despite the wealth of available germplasm amongst the Iridaceae, only 40 species from 12 genera have been micropropagated (Ascough et al., 2009). George (1993) and Ascough et al. (2009) presented extensive summaries of bulbous and cormous species which were

micropropagated *in vitro,* and subsequently successfully hardened-off and established *ex vitro*. According to Ascough et al. (2007), root and leaf explants of *Watsonia* spp. were incapable of shoot regeneration, but hypocotyl segments were highly regenerative when both an auxin (NAA) and cytokinin (BAP) were present in the medium. However, shoot multiplication was greatest when only BAP (2.2 µM) was added to the medium. Similar results were obtained for *Dierama latifolium* (Page and van Staden, 1985) and *Schizostylis coccinea* (Hussey, 1976), both iridaceous species. In the current study, aseptic cultures of *C. aurea* were readily established using the methods described above. Although BAP at a concentration of 4.4 µM had a tendency to produce a slightly higher multiplication rate (3.35 shoots every 4-6 weeks) than BAP at a concentration of 8.8 µM (3.2 shoots every 4-6 weeks), the difference in treatments was not significant after a period of 4 months. The addition of BAP at a concentration of 13.2 µM gave a shoot multiplication rate of 2.75. Although growth regulator addition usually speeds up the rooting process and increases rooting percentage, as has been reported for several other genera (George, 1993), transfer of *C. aurea* shoots to a growth regulator-free MS medium resulted in root formation on 100% of shoots cultured in this experiment.

3.2. Germination rate of colchicine-treated seeds

The germination rate and percentage of seeds treated with 0.25 µM colchicine tended to be higher than that of the 25 µM treatment ($p < 0.05$), although the difference was not statistically significant (Fig 1). However, colchicine had a significant negative effect on germination for both the 0.25 µM overnight and 25 µM three day treatments, when compared with the control. The differences in germination rate manifested five to six weeks after initial exposure to colchicine. Similar findings have been reported on a wide range of species (Ramsey and Schmeske, 1998).

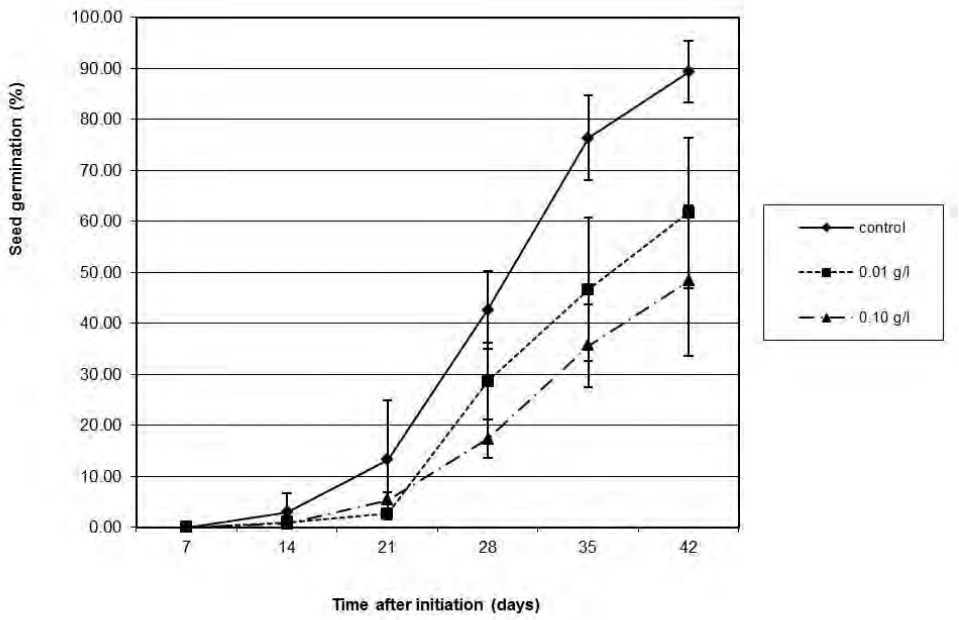


Fig 1. Effect of colchicine concentration on seed germination of *Crocosmia aurea*.

3.3. Effect of colchicine on polyploidy induction and in vitro development of induced plantlets

Samples of leaf material were harvested for flow cytometry analysis from *in vitro*-grown shoots 12 weeks after colchicine treatment. Flow cytometry analysis was carried out to determine the ploidy levels of *in vitro* regenerants, which included mixoploids (Fig. 2). Of the plants treated overnight with 0.25 µM colchicine, 29.82% were identified as tetraploids, with 8.77% identified as 2n:4n mixoploids and 3.51% as 4n:8n mixoploids (Table 1). A small percentage (4.39%) was identified as octoploid for the same treatment. For the 25 µM three-day treatment, 16.04 % of the seedlings were identified as tetraploid, with 9.8% being 2n:4n mixoploids and no octoploids identified (Table 1). All mixoploids were discarded to prevent proliferation of an unstable population of such plants. The effectiveness of colchicine application and polyploidy induction *in vitro* not only depends highly on the plant species but also on the colchicine concentration applied, duration of treatment, type of explant, and the penetration of the compound (Allum et al., 2007). Colchicine has been used effectively at both lower (i.e. 0.25 µM for *Lychnis senno* (Chen et al., 2006)) and very high concentrations (i.e. 38,000 µM for *Chaenomeles japonica* (Stanys et al., 2006)). Within the Iridaceae family, polyploidy has been successfully induced in *Gladiolus* spp. (Suzuki et al., 2005) and *Watsonia lepida* (Ascough et al., 2008).

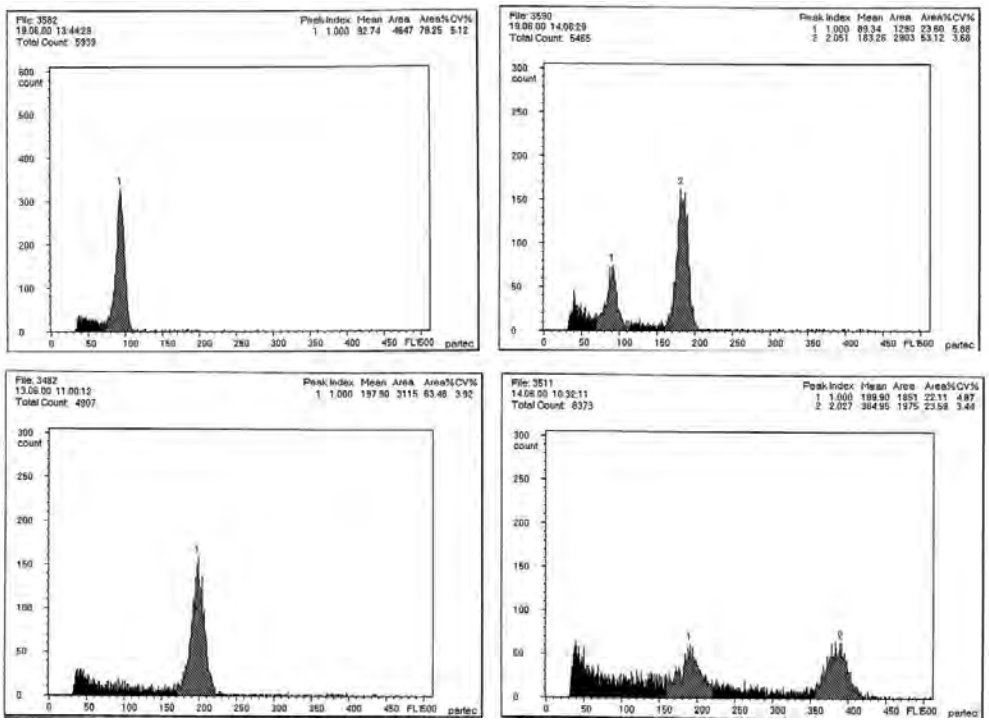


Fig 2. Representative flow cytometric histograms documenting the ploidy level of nuclei isolated from leaves. A. diploid; B. tetraploid, C. diploid:tetraploid mixoploid, D. tetraploid:octoploid mixoploid.

Table 1. Effect of *in vitro* colchicine treatments on polyploidy induction in *Crocosmia aurea.*

Treatment	Exposure time	Ploidy (%)				
		diploids	2n:4n mixoploids	tetraploids	4n:8n mixoploids	octoploids
Control		100.0	0	0	0	0
0.25 µM	overnight	53.51	8.77	29.82	3.51	4.39
25 µM	3 days	74.16	9.80	16.04	0	0

There was no significant difference in *in vitro* shoot height, multiplication rate or rooting between *in vitro* diploid and tetraploid shoots (Table 2). Rooted plantlets, both diploid and tetraploid, were readily hardened-off in the mistbed. There was no difference in survival rate or in plant growth and development between diploid and tetraploid plants. Survival rate was in the order of 98% for both diploid as well as for tetraploid plants.

Table 2. Comparison after 4 months of the *in vitro* performance of diploid and tetraploid *Crocosmia aurea* plantlets on MS nutrient medium supplemented with 30g/l sucrose and various concentrations of 6-benzylaminopurine (BAP). Different letters denote a significant difference at $p < 0.05$.

Treatment	0 µM BAP		4.4 µM BAP		8.8µM BAP		13.2 µM BAP	
	diploid	tetraploid	diploid	tetraploid	diploid	tetraploid	diploid	tetraploid
Proliferation rate*	2.13a	2.25a	3.35b	3.25b	3.20b	3.32b	2.75a	2.63a
Shoot height (mm)	45.2a	42.3a	46.5a	45.3a	42.5a	41.8a	43.5a	42.1a
Rooting (%)	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a

*number of shoots per 4-6 week subculture interval

3.4. Morphological characteristics of tetraploid C. aurea plants

Overall flower size i.e. flower diameter, petal width, petal length, stamen length, diameter and length of the inflorescence stem as well as the number of flowers on the inflorescence were significantly different for the induced tetraploid plants compared with the diploid plants (Table 3). Overall flower size i.e. flower diameter and petal length and breadth (Fig. 3), as well as the diameter of the inflorescence stem, were significantly larger and thicker, respectively, for the induced tetraploids compared with the diploids. Conversely, the length of the inflorescence stem and number of flower buds per inflorescence was significantly lower for the induced tetraploids. Polyploidy can result in significant cell enlargement, which is particularly desirable for flowering ornamental species. Several reports since the advent of induced polyploidy research on ornamentals describe increases in flower size (Emsweller and Ruttle, 1941; Tulay and Unal, 2010), alterations in inflorescence stem length (Griesbach and Bhat, 1990; Takamura and Miyajima, 1996), and number of flowers per stem (Kafawin and Chen, 1991; Tulay and Unal, 2010) for a wide range of ornamental species, and with differing results. There is no means to predict direction of morphological or physiological change for induced polyploids for a particular species. For *C. aurea*, there appeared to be no significant difference between induced tetraploids and diploids regarding certain reproductive parts of the flower (anther and stigma length), while others (stamens) were significantly longer in the tetraploids than in the diploids. The induced tetraploids appeared to be sterile since no seed was set over the

evaluation period compared with the diploids, 100% of which set seed. This could be a physical constraint during pollination due to the altered morphology of the plant parts, since pollen viability studies using acetocarmine staining showed no difference in viability between diploid and tetraploid pollen (results not shown). Changes in flower shape, due to polyploidy, have been reported to attract different species of insects to the polyploid flowers which could preclude pollination (Thompson and Merg, 2008). Although tetraploid plants did not set seed, the *in vitro* micropropagation method developed would facilitate the establishment of a mother-block of polyploid plants (and thereby establish a genebank) which are easily hardened-off. Producers could then successfully vegetatively propagate *C. aurea* through division of corms.

Table 3. Effect of polyploidy on selected morphological characteristics of *Crocosmia aurea*. (different letters within a row denote significant difference at $p<0.05$, n= 12).

Morphological characteristic	diploid	tetraploid
Flower diameter (mm)	62.78a	78.2b
Width of petal (mm)	8.936a	15.04b
Length of petal (mm)	31.05a	36.72b
Length of stamens (mm)	26.07a	32.27b
Length of stigma (mm)	29.70a	29.03a
Length of anther (mm)	9.53a	8.585a
Diameter of inflorescence stem (mm)	3.09a	3.54b
Length of inflorescence stem (mm)	794.67b	577.70a
Number of flowers	29.00b	21.67a

Fig 3. Comparison of diploid (A and B) and induced tetraploid (C and D) *C. aurea* flowers. Scale bar represents 1cm.

C. aurea is thus readily propagated *in vitro*, and *in vitro* tetraploid induction provides a method for the development of *C. aurea* plants with significantly larger flowers than their diploid counterparts. Preliminary observations of flowering of the tetraploid plants indicated that

flowering appears to be initiated several weeks after the diploids. In fact, flowering mimics the vegetative phenological cycle, with the dormant tetraploid corms producing vegetative growth several weeks after the diploids (preliminary observation; data not shown). It has previously been reported that tetraploids, which may have a slower growth rate, may flower later or over a longer period than their diploid progenitors (Datta, 1963; Roy and Dutt, 1972; Levin, 1983). This is an attribute of particular interest in ornamental breeding (Weiss, 2002). Furthermore, Kehr (1996) reported that flowers are longer lasting in polyploid plants – vase life has yet to be determined for the tetraploid plants produced in this study. In conclusion, additional research is needed to establish possible alterations in vase-life of tetraploids compared with the diploids; phenological studies to determine flowering times are also required to determine a potential increase or decrease of marketing windows.

Acknowledgements

The Agricultural Research Council and the Department of Science and Technology are thanked for funding the investigations. The assistance of Mr Mark Penter with proofreading is acknowledged. Mr Gerrit Visser is thanked for conducting the flow cytometry.

References

Allum, J.F., Bringloe, D.H., Roberts, A.V., 2007. Chromosome doubling in *Rosa rugosa* Thunb. hybrid by exposure of *in vitro* nodes to oryzalin: The effects of node length, oryzalin concentration and exposure time. Plant Cell Reports 26, 1977–1984.

Ascough, G.D., Erwin, J.E., van Staden, J., 2007. *In vitro* propagation of four *Watsonia* species. Plant Cell Tissue and Organ Culture 88, 135–145.

Ascough, G.D., Erwin, J.E., van Staden, J., 2008. Effectiveness of Colchicine and Oryzalin at Inducing Polyploidy in *Watsonia lepida* N.E. Brown. Plant Cell Tissue and Organ Culture 43, 2248-2251.

Ascough, G.D., Erwin, J.E., van Staden, J., 2009. Micropropagation of iridaceae - a review. Plant Cell Tissue and Organ Culture 97, 1–19.

Caperta, A.D., Delgado, M., Ressurreição, F., Meister, A., Jones, R.N., Viegas, W., Houben, A., 2006. Colchicine-induced polyploidization depends on tubulin polymerization in c-metaphase cells. Protoplasma 227, 147–153.

Chen, L.P., Wang, Y.J., Zhao, M., 2006. *In vitro* induction and characterization of tetraploid *Lychnis senno* Siebold et Zucc. HortScience 41, 759–761.

Datta, R.M., 1963. Investigations on the autotetraploids of the cultivated and the wild types of jute (*Corchorus olitorius* Linn. and *C. capsularis* Linn.). Zuchter 33, 17-33.

Emsweller, S., Ruttle, M., 1941. Induced polyploidy in floriculture. American Naturalist 75, 310-328.

Galbraith, D.W., Lambert, G.M., Macas, J., Doležel, J., 1997. Current protocols in cytometry, Analysis of nuclear DNA content and ploidy in higher plants, ed Robinson J.P. (John Wiley and Sons, New York), pp 7.6.1–7.6.22.

George, E., 1993. Plant Propagation by Tissue Culture, second ed., Exegetics Limited, Edington, England.

Griesbach, R., Bhat, R., 1990. Colchicine-induced polyploidy in *Eustoma grandiflorum*. HortScience 25, 1284-1286.

Hussey, G., 1976. *In vitro* release of axillary shoots from apical dominance in monocotyledonous plantlets. Annals of Botany 25, 1323–1325.

Kafawin, O., Chen, C., 1991. Induction of Tetraploid *Lilium longflorium* Thumb Plants By Colchicine Treatment of Cultured Bulbscale Discs. Proceedings of the South Dakota Academy of Science 70, 31-37.

Kehr, A.E., 1996. Woody plant polyploidy. American Nurseryman 183, 38-47.

Koh, J.C., Lee, E.J., Park, H.G., 2007. Effects of several plant growth regulators on callus and corm formation of *Crocosmia crocosmiiflora in vitro*. Flower Research Journal 15, 238–244.

Levin, D., 1983. Polyploidy and novelty in flowering plants. American Naturalist 122, 1-25.

Murashige, T., Skoog, F., 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15, 473–497.

Niederwieser, J.G., Kleynhans, R., Hancke, F.L., 2002. Development of a new flower bulb crop in South Africa. Acta Hortiulturae 570, 67–71.

Page, Y.M., van Staden, J., 1985. *In vitro* propagation of *Dierama latifolium*. HortScience 20, 1049–1050.

Pooley, E., 1993. The Complete Field Guide to the Trees of Natal, Zululand and Transkei. KZN Flora & Fauna Publications Trust, KwaZulu-Natal.

Ramsey, J., Schmeske, D., 1998. Pathways, mechanisms and rates of polyploidy formation in flowering plants. Annual Review of Ecology and Systematics 29, 467-501.

Roy, R.P., Dutt, B., 1972. Cytomorphological studies in induced polyploids of *Luffa acutangula* Roxb. Nucleus 15, 181-189.

Stanys, V., Weckman, A., Staniene, G., Duchovskis, P., 2006. *In vitro* induction of polyploidy in Japanese quince (*Chaenomeles japonica*). Plant Cell Tissue and Organ Culture 84, 263–268.

Suzuki, K., Takatsu, Y., Gonai, T., Kasumi, M., 2005. Plant regeneration and chromosome doubling of wild *Gladiolus* species. Acta Horticulturae 673, 175–181.

Takamura, T., Miyajima, I., 1996. Colchicine-induced tetraploids in yellow-flowered cyclamens and their characteristics. Scientia Horticulturae 65, 305-312.

Thompson, J.N., Merg, K.F., 2008. Evolution of polyploidy and the diversification of plant–pollinator interactions. Ecology 89, 2197-2206.

Tulay, E., Unal, M., 2010. Production of colchicine-induced tetraploids in *Vicia villosa* Roth. Caryologia 63, 292-303.

Väinölä, A., 2000. Polyploidization and early screening of *Rhododendron* hybrids. Euphytica 112, 239–244.

Weiss, D., 2002. Introduction to new cut flowers: domestication of new species and introduction of new trait not found in commercial varieties, in: Vainstein, A. (ed.), Breeding for Ornamentals: Classical and Molecular Approaches. Kluwer Academic Publishers, Netherlands, pp. 129-137.

Yang, X., Ye, C-Y., Cheng, Z-M., Tschaplinski, T.J., Wullschleger, S.D., Yin, W., Xia, X., Tuskan, G.A., 2011. Genomic aspects of research involving polyploid plants. Plant Cell Tissue and Organ Culture 104, 387-397.

CHAPTER 4

In vitro-induced polyploidy and its effect on horticultural characteristics, essential oil composition and bioactivity of *Tetradenia riparia.*

K. Hannweg[a,b*], G. Visser[a], K. de Jager[a] and I. Bertling[b]

[a] Agricultural Research Council-Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, 1200, South Africa

[b] Horticultural Science, School of Agricultural, Earth and Environmental Sciences, Private Bag X01, University of KwaZulu-Natal, Scotsville, 3209, South Africa

(email address of corresponding author: Karin@arc.agric.za)

Submitted and under review for publication in South African Journal of Botany.
Impact factor: 0.978 (2015)

This manuscript is formatted according to the 2015 formatting requirements of the South African Journal of Botany.

In vitro-induced polyploidy and its effect on horticultural characteristics, essential oil composition and bioactivity of *Tetradenia riparia.*

K. Hannweg[a,b*], G. Visser[a], K. de Jager[a] and I. Bertling[b]

[a] Agricultural Research Council-Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, 1200, South Africa

[b] Horticultural Science, School of Agricultural, Earth and Environmental Sciences, Private Bag X01, University of KwaZulu-Natal, Scotsville, 3209, South Africa

(email address of corresponding author: Karin@arc.agric.za)

Abstract

Tetradenia riparia, a medicinal aromatic shrub with multiple uses, occurs throughout tropical Africa and as far south as South Africa. *In vitro*-induced polyploidy of *T. riparia* was carried out by treating seeds with colchicine and subsequently verifying the duplication of chromosomes using flow cytometry. Significant differences between diploid and tetraploid plants in terms of leaf morphology, essential oil content and characterisation as well as *in vitro* bioactivity against *Geotrichum candidum*, a fungal species causing postharvest rot in fruit and vegetables, were recorded. Tetraploid plants produced essential oil with the potential for mitigating postharvest diseases. This is the first report on the bioactivity of *T. riparia* essential oil against a plant pathogenic fungal species of postharvest concern.

Keywords: bioactivity, essential oil, induced polyploidy, plant characteristics

1. Introduction

Tetradenia riparia, commonly referred to as 'ginger bush', is an aromatic shrub occurring throughout tropical Africa and the north eastern regions of South Africa. It has a range of medicinal uses (Coopoosamy and Naidoo, 2011) and is also used widely as a flavouring agent as well as in the perfume industry. The major chemical components of interest include the diterpenes, alpha-pyrones and phytosterols, as well as essential oils comprising a number of compounds. Previously reported essential oil bioactivity includes moderate anti-malarial activity against *Plasmodium falciparum* (Campbell et al., 1997), anti-spasmodic activity (Van Puyvelde et al., 1987), antiviral activity, antimicrobial activity against various human pathogenic bacteria (Boily and Van Puyvelde, 1986; Van Puyvelde et al., 1994; Ndamane et al., 2013) as well as insecticidal (Weaver et al., 1992) and acaricidal activity (Gazim et al., 2011).

To our knowledge there has been no genetic improvement, breeding or selection of *Tetradenia riparia*. Induced polyploidy has been reported to result in the improvement, although not always, of a range of horticultural characteristics including larger tuber, rhizome or root size; increased fruit size; enhanced flower size and/or colour intensity, improved drought tolerance, increased biomass; improved photosynthetic capacity; larger and/or thicker leaves; dwarfism and increased secondary metabolite production e.g. medicinal compounds (Ranney, 2006, Caruso et al., 2011). Chromosome doubling (polyploidy) has, moreover, been reported to have significant effects on not only secondary metabolite (e.g. essential oil) yield but also secondary metabolite profiles across diverse species including *Petunia* (Griesbach and Kamo, 1996); *Chamomill arecutita* (Svehlikova and Repcak., 2008); *Artemisia annua* (De Jesus-Gonzalez and Weathers, 2003) and *Ocimum basilicum* (Omidbaigi et al., 2010). This topic has also been

reviewed by Dhawan and Lavania (1996). There is also widespread interest in the potential applications of bioactive phytochemicals of aromatic and medicinal plants which have been shown to exhibit anti-microbial, anti-fungal and insecticidal properties, as they have the potential for substituting synthetic chemicals which are often detrimental to the environment. Further, Dhawan and Lavania (1996) and Lavania (2005) suggested that artificial polyploids could be used to achieve commercial impact with respect to the production of secondary metabolites. The current investigation reports on methods for polyploidy induction and verification, a comparison of selected horticultural characteristics between the diploid progenitor and induced tetraploids, as well as a first report on the bioactivity of the induced tetraploid-derived essential oil against a fungal species (*Geotrichum candidum*) of postharvest importance.

2. Materials and Methods

2.1 *In vitro* polyploid induction

Tetradenia seeds obtained from Silverhill Seeds (Cape Town, South Africa) were surface-sterilised in 1% [v/v] calcium hypochlorite before being rinsed three times using autoclaved distilled water. The sterilized seeds, one hundred per treatment, were submerged in colchicine solutions, either 25 µM or 250 µM, overnight or for three days, respectively. These combinations of colchicine concentration and incubation time have proven successful for polyploid induction in a number of other species studied in this laboratory (Hannweg *et al*., 2013; Hannweg *et al.*, 2015). Following treatment, seeds were cultured individually in glass culture tubes containing 10 ml Murashige and Skoog [MS] (1962) medium (nutrients and vitamins) supplemented with 30 g l^{-1} sucrose and 3 g l^{-1} Gelrite®. Seeds were germinated at 25ºC under a 16:8 hour light:dark regime (cool white fluorescent light; 81 µmol m^{-2} s^{-1}, Phillips

65W). The seedlings were large enough for ploidy analysis using flow cytometry after 10-12 weeks.

2.2 Analysis of ploidy level using flow cytometry

Samples of leaf material were harvested for flow cytometry analysis from *in vitro*-grown shoots. The ploidy level of treated seedlings was verified using a Partec PA ploidy analyser (Partec, Germany). Samples were prepared for flow cytometry analysis using approximately 0.5 cm^2 leaf tissue. The tissue was macerated with a razor blade in 125 µl nucleus extraction solution (Partec, Germany), after which the homogenate was filtered through a 50 µm mesh filter. The isolated nuclei were stained with 1250 µl 4'-6-diamidino-2-phenylindole (DAPI) stain (Partec, Germany) prior to commencing flow cytometry analysis. Nuclei isolated from untreated, diploid plantlets were used as a standard. Histograms were analysed using the Partec software package. Seedlings confirmed as tetraploid were proliferated on the MS medium described above, supplemented with 4.4 µM 6-benzylaminopurine (BAP) under the same conditions. Rooting occurred spontaneously *in vitro*, and rooted plantlets were readily acclimatised in the mistbed with a 5 second misting per hour. Acclimatised plants were planted initially into 2 l potting bags containing a 1:1 mixture of pine bark and sand for plant establishment before being transplanted into 20 l bags for establishment and maintenance in a shadehouse (40% black shade net). Plants were drip-irrigated and Osmocote® slow-release granules were applied to the potting medium at the manufacturer's recommended rate.

2.3 Characterisation of selected morphological characteristics, essential oil and bioactivity of tetraploids

To determine if there were differences in selected morphological characteristics, established diploid control plants and tetraploid plants were evaluated for plant stature, leaf shape (leaf and leaf margin morphology) and petiole length as well as essential oil yield and quality (n=12 each of diploid and tetraploid plants). Essential oils were extracted by steam-distillation using a Clevenger-type apparatus for 1 hour using freshly-harvested leaves, and essential oil yield determined on a fresh weight basis. The essential oils were stored in amber vials at 4 ºC. Major constituents were analysed using gas chromatography and mass spectrometry (GC-MS), using standard methods (Tshwane University of Technology, Pretoria, South Africa). Bioactivity of the essential oils derived from diploid and tetraploid plants against *Geotrichum candidum* was investigated by isolating the fungus from cucumber and culturing on potato dextrose agar (PDA, LABM, United Kingdom) medium in 90 mm Petri dishes before being used to produce fungal spore dilutions. No result was obtained when the disc diffusion method was used to determine bioactivity and since the limited volume of the essential oil available precluded an investigation of zones of inhibition, fungal spores and essential oils were incubated together before being cultured on PDA medium as follows: Fungal spores at three different concentrations, determined using a haemocytometer, and essential oils (0, 100, 200, 500 and 1 000 ppm) were incubated for 1, 6 h and 12 h, at ambient temperature. The essential oil-fungal spore solutions were well-mixed for three seconds using a bench-top vortex prior to incubation. Since the pathogen has a relatively short disease cycle, the incubated mixtures were inoculated and cultured on PDA medium in 90 mm Petri dishes and fungal colonies counted over a period of one week to determine potential bioactivity. Petri dishes containing PDA and inoculated with the fungal spore suspensions only were used as controls. EC50 values were also

determined across incubation time, number of fungal spores and diploid- compared with tetraploid-derived essential oils.

2.4 Statistical analysis

For the polyploid induction/colchicine applications, an appropriate analysis of variance was fitted to the data using PROC GLM procedure of SAS software Version 9.2 of the SAS System for Windows (SAS Institute, 2015). The Shapiro-Wilk test was performed to test for normality (Shapiro and Wilk, 1965) and a Fisher t-test with Least Significant Difference was calculated at the 5% significance level to compare treatment means (Ott and Longnecker, 2001). For all other experiments, the experimental layout for all experiments was a complete randomised design (CRD) with 3 replications. An analysis of variance (student's t-test) was performed at the 1% significance level for these experiments except the EC50 values where various non-linear regression models (Gompertz, sigmoid, natural growth, modified exponential and logistic regression) were fitted to the data, with the natural growth model being the best fit. Observations where there was no variation in response to the various concentrations were removed from the data set before the ANOVA was performed (SAS Institute, 2015).

3. Results and discussion

3.1 Polyploid induction and verification

Colchicine is a highly-effective mitotic spindle inhibitor and has been used with great success for the improvement of horticultural characteristics for a variety of species via polyploidy induction (Dhooghe et al., 2011). Polyploid induction has successfully been used with other species rich in economically-useful essential oils (Table 1) and has considerable potential to

alter both oil quantity and the profile of its components. As such, this study was initiated to determine the effect of induced polyploidy on various characteristics of *Tetradenia riparia*, including its oil profile. Following colchicine treatment, it is critical to identify plant lines in which polyploidy has successfully been induced. Flow cytometry provides a quick, reliable method for determination of ploidy level and is thus an efficient means of screening large populations emanating from plant improvement programmes (Ochatt et al., 2011). In this study, flow cytometry analysis was carried out to determine the ploidy levels of *in vitro* regenerants, which included mixoploids (Table 2). For the 25 µM overnight treatment, 61% of shoots remained diploid, 25.6% of shoots were verified as pure tetraploids and 13.4% were identified as 2n:4n mixoploids. For the 250 µM, 3 day treatment, 18.0% of the shoots were verified as tetraploids, 72.1% were not transformed and remained diploid, while 9.9% were identified as 2n:4n mixoploids. No octoploids or 4n:8n mixoploids were identified for either of the treatments, in contrast to Hannweg et al. (2013) where significant levels of these were induced. All mixoploids were discarded to prevent the multiplication of unstable plant populations. Efficacy in ploidy transformation is dependent on exposure time, colchicine concentration and plant species and successful polyploid induction is unpredictable.

Table 1. Important crop plant species where essential oils have been improved using polyploidy induction.

Scientific name	Common name	Family	Reference
Vetiveria zizanioides	Vetiver	Poaceae	Lavania, 1988
Ocimum kilimandscharicum	Camphor basil	Lamiaceae	Bose and Choudhury, 1962
Carum carvi	Caraway	Apiaceae	Dijkstra and Speckman, 1980
Mentha arvensis	Jammu mint	Lamiaceae	Janaki Ammal and Sobti, 1962
Humulus lupulus	Hops	Cannabaceae	Koutoulis et al., 2005
Ocimum basilicum	Basil	Lamiaceae	Omidbaigi et al., 2010
Acorus calamus	Bacha	Araceae	Pattanaik et al., 2013
Mentha piperita	Peppermint	Lamiaceae	Rita and Animesh, 2011
Chamomilla recutita	Chamomile	Asteraceae	Svehlikova and Repcak, 2008
Aframomum corrorima	Korarima	Zingiberiaceae	Wannakrairoj and Wondyifraw, 2013
Zingiber officinale	Culinary Ginger	Zingiberiaceae	Wohlmuth et al., 2005

Table 2. Effect of *in vitro* colchicine treatments on polyploidy induction in *Tetradenia riparia*.

Treatment ($g.l^{-1}$)	Exposure time	Ploidy (%)				
		diploids	**2n:4n mixoploids**	**tetraploids**	**4n:8n mixoploids**	**octoploids**
Control		100.0	0	0	0	0
0.01	overnight	61.0	13.4	25.6	0	0
0.10	3 days	72.1	9.9	18.0	0	0

3.2 Characterisation of tetraploid *Tetradenia riparia* plants

3.2.1 Morphological characteristics

Although there were no obvious differences in plant height, (and no difference in internode length) between induced tetraploid and diploid plants (results not shown), the induced tetraploid plants were characterized by thicker and stickier leaves compared with the diploid plants. Leaves of the induced tetraploids were also more rounded in shape while the leaves of the diploids were cordiform (heart-shaped) (Figure 1). Further, the induced tetraploid plants had highly-lobed leaf margins compared with the diploids. Tetraploid leaf petioles were significantly shorter than the diploids (on average, 20 mm compared with 31 mm, respectively).

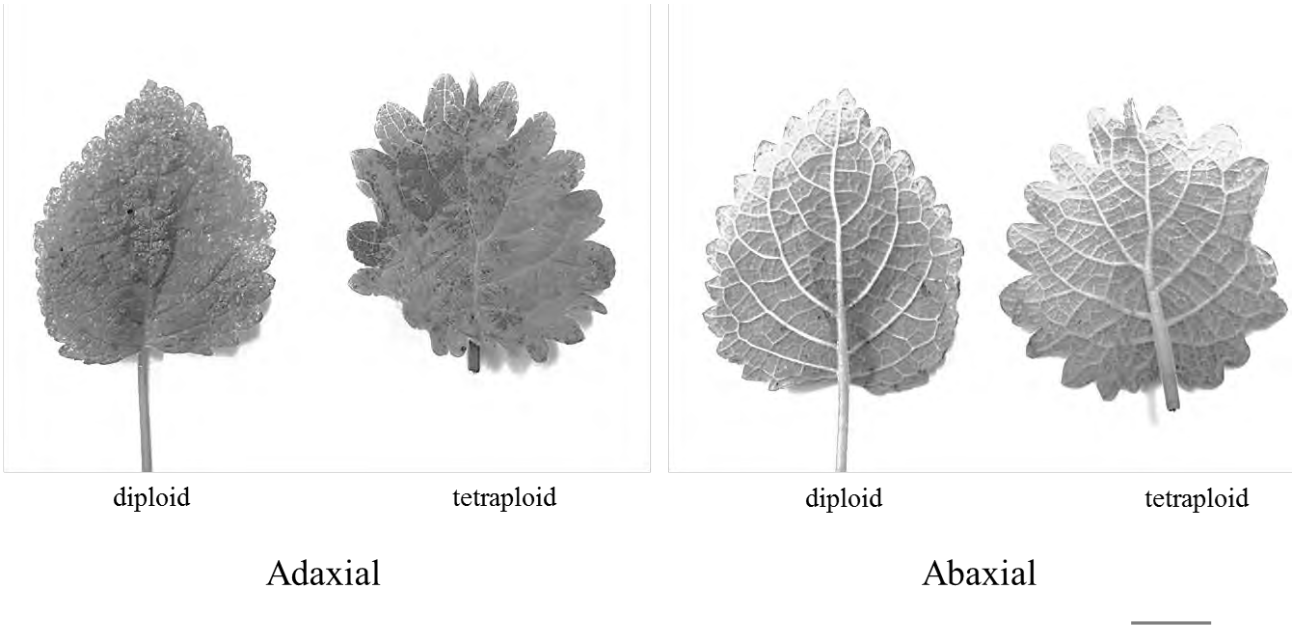


Fig 1. Morphological comparison between leaves from diploid and tetraploid plants showing adaxial (L) and abaxial (R) leaf shape, leaf surfaces and deeply dentate leaf margins (scale bar: 1cm = 1.5 cm).

3.2.2 Comparison of essential oil characteristics between diploid and tetraploid *Tetradenia riparia* plants

Tetraploid plants produced three and a half times more oil than diploid plants on a fresh weight basis (0.25% compared with 0.07%) and, interestingly, the essential oil derived from the leaves of tetraploid plants was colourless compared with the yellow essential oil distilled from leaves from diploid plants, probably due to differences in the oils' component compounds. This increase in oil content is consistent with several plant species producing greater quantities of important secondary metabolites, as reviewed by Lavania (2005). When oil samples were analysed for their major constituents, marked differences between certain components in the tetraploid- and diploid-derived essential oils were revealed (Table 3). Tetraploids contained significantly more fenchone than diploids, and tetraploids contained alpha-humulene, alpha-

terpineol and viridiflorol which were not present in the diploids. Conversely, the diploid essential oils contained fenchyl acetate and large amounts of isopimara-8,15-diene, which were not present, or present in extremely low concentrations respectively, in the tetraploid samples (Table 3). Induced polyploidy in essential oil crops was comprehensively reviewed by Dhawan and Lavania (1996). In this review, the authors describe a number of studies on essential oil-bearing plant species in which induced tetraploids and diploids had significantly different secondary metabolite profiles from each other, as found in this study. The alteration of components of the essential oil profiles could be ascribed to changes in the regulation of synthesis of biochemicals (Lavania, 2005). Further, Levy (1976) suggested that a reduction or even loss of components could be due to functional repression of genes, while gains are ascribed to derepression of silent genes previously not expressed.

Table 3. Comparison between major chemical components of essential oils distilled from diploid and induced tetraploid plants[§]

Component	Diploid	Tetraploid
alpha-pinene	0	2.26
camphene	0	0.76
limonene	1.84	1.68
gamma-terpinene	1.74	0
terpinolene	0	1.32
p-Cymene	0.72	0
fenchone	**25.42**	**48.4**
fenchyl actetate	**16.68**	**0**
alpha-Copaene	2.74	0
beta-Bourbonene	1.04	0
camphor	0.86	2.68
fenchol	0	1.4
bornyl acetate	2.02	0
beta-caryophyllene	1.34	0
terpinen-4-ol	0.62	0
alpha-humulene	**0**	**7.92**
alpha-terpineol	**0**	**10.16**
germacrene D	2.46	0
bicyclogermacrene	0.84	0
delta-Cadinene	1.26	0.82
viridiflorol	**0**	**5.58**

cubedol	0.86	1.22
caryophyllene oxide	0.5	0
thymol	3.14	0
unknown compound	1.9	1.36
isopimara-8,15-diene*	**14.44**	**0.8**
abietatriene*	0.74	0.12

[§]Only components where the component percentage was ≥0.5 have been included in the Table.
[¥]Where there are major differences between components, these have been highlighted in bold.
*Components denoted by * were identified using mass spectrometry only

3.2.3 Bioactivity of essential oil against *Geotrichum candidum*

The effect of polyploidy on the bioactivity of the essential oils steam-distilled from diploid and tetraploid leaves was tested against *Geotrichum candidum*, a fungal species of postharvest concern which occurs during storage of fruits and vegetables. Bioactivity of the oils increased with incubation time and essential oil concentration (Table 4), as evidenced by a corresponding decrease in the number of fungal colonies. The number of colonies counted was also significantly lower when spores were incubated with essential oil derived from the induced tetraploids compared with the essential oil of the diploids. Analysis of the EC50 values, a measure of the effectiveness of the essential oil, indicated that a one hour incubation time was not significant across the treatments and did not have any effect on fungal spore germination and was therefore ineffective under these conditions (Table 5). However, essential oils derived from the induced tetraploids had a more pronounced effect at each spore concentration tested in this study compared with the diploids at both the six hour and twelve hour incubation times and this was highly significant. α-Terpineol and viridiflorol, the major constituents known to have anti-fungal properties (Tabassum and Vidyasagar, 2013), were present only in the tetraploid oil and not in the diploid oil. The bioactivity of the oil derived from tetraploid

individuals may be due to the presence of these compounds. Further, Dhawan and Lavania (1996) stated that where changes in constituent profile occur as a result of chromosome doubling, there may be an effect on the biochemical pathway of the relevant constituent's biosynthesis and as alluded to in Section 3.2.2 above, could be controlled genetically. This is yet to be elucidated for *Tetradenia riparia* regarding the constituents involved in anti-fungal activity.

This is the first published report describing the bioactivity of *Tetradenia riparia* essential oils against a plant pathogen. The essential oils derived from tetraploid plants appear to have a higher bioactivity against *G. candidum* than essential oils derived from diploid plants. The use of essential oils as amendments to or alternatives to chemical fungicides is a relatively new approach to the control of postharvest pathogens (Klieber et al., 2002, Ahmed et al., 2007, du Plooy et al., 2009; Kouassi et al., 2010). The use of natural products is environmentally-friendly and would allow organic producers to use such a product, as it is likely to meet the organic requirements, result in reduced environmental concerns and greater consumer acceptance while providing significant disease control. Further studies into the application of the essential oils to products susceptible to *G. candidum* under postharvest storage conditions needs to be investigated and is continuing. The essential oils should also be tested for their possible efficacy against other pathogens of postharvest concern and the effect of induced polyploidy on the mechanisms of biochemical pathway alteration should also be investigated.

Table 4. Comparison of fungal colony number after incubation of *Geotrichum candidum* with different concentrations (0-1000ppm) of tetraploid- and diploid-derived essential oil of *Tetradenia riparia* with increasing incubation time. Means with the same letter are not significant.

	1 hour						6 hours						12 hours					
	diploid			tetraploid			diploid			tetraploid			diploid			tetraploid		
No. of spores/ml	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7
Concentration																		
0 ppm	91^{O}	1500^{A}	1500^{A}	200^{M}	1200^{B}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}	1500^{A}
100 ppm	68^{P}	1500^{A}	1500^{A}	225^{K}	1200^{B}	1500^{A}	60^{Q}	500^{D}	1500^{A}	12^{X}	350^{G}	1500^{A}	50^{R}	320^{H}	1500^{A}	15^{W}	500^{D}	1000^{C}
200 ppm	38^{S}	1500^{A}	1500^{A}	32^{T}	900^{C}	1500^{A}	20^{V}	500^{D}	1500^{A}	8^{Y}	350^{G}	1500^{A}	10^{XY}	360^{F}	1500^{A}	4^{aZ}	280^{I}	1500^{A}
500 ppm	58^{Q}	1500^{A}	1500^{A}	49^{R}	480^{E}	1500^{A}	59^{Q}	500^{D}	1500^{A}	11^{XY}	350^{G}	1500^{A}	15^{X}	250^{J}	1500^{A}	4^{abZ}	208^{L}	1500^{A}
1000 ppm	4^{aZ}	1500^{A}	1500^{A}	5^{Z}	27^{U}	1500^{A}	16^{W}	500^{D}	1500^{A}	0^{c}	350^{G}	1500^{A}	3^{abc}	150^{N}	1500^{A}	5^{bc}	63^{Q}	1500^{A}

Table 5. Effectiveness of diploid- and tetraploid-derived essential oils of *Tetradenia riparia* against *Geotrichum candidum* was calculated after incubation for 1 hour, 6 hours and 12 hours with increasing fungal spore concentrations. Means with the same letter are not significant.

Incubation time	**1 hour**						**6 hours**						**12 hours**					
Essential oil	**diploid**			**tetraploid**			**diploid**			**tetraploid**			**diploid**			**tetraploid**		
No. of spores/ml	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7	2.4×10^5	2.4×10^6	2.4×10^7
EC_{50}[w] (ppm)	420.2c	*	*	*	442.9a	*	266.7g	420.8b	*	261.1i	354.8d	*	262.1h	313.0e	*	260.7j	298.9f	*

*Indicates no effect on spore viability

[w] Essential oil concentration estimated to produce 50% inhibition.

4. Acknowledgements

The Agricultural Research Council (ARC) and Department of Science and Technology are thanked for grant funding. The Tshwane University of Technology, Pretoria, South Africa, is acknowledged for co-operation with the GC-MS analyses of the essential oils. Mardé Booyse and Mzabalazo Ngwenya of the ARC-Biometry Unit are thanked for their assistance with the statistical analysis.

5. References

Ahmed, D., El-Shami, S. and El-Mallah, M. 2007. Jojoba oil as a novel coating for exported Valencia orange fruit. Part 1. The use of trans (isomerized) jojoba oil. American-Eurasian. Journal of Agricultural and Environmental Sciences 2, 173–181.

Boily, Y, and van Puyvelde, L. 1986. Screening of medicinal plants of Rwanda for antimicrobial activity. Journal of Ethnopharmacology 16: 1-13.

Bose, R. and Choudhury, C. 1962. A Comparative Study of the Cytotaxonomy, Pallynology, Physiology of 'Diploid' and 'Polyploid' Plants of *Ocimum kilimandscharicum* Guerke and their Yield of Raw Material and Volatile Contents. Caryologia 15, 435-454.

Campbell, W., Gammon, D., Smith, P., Abrahams, M. and Purves, T. 1997. Composition and anti-malarial activity *in vitro* of the essential oil of *Tetradenia riparia.* Planta Medica 63, 270-272.

Caruso, I., Lepore, L., De Tommasi, N., Dal Piaz, F., Frusciante, L., Aversan, R., Garramone, R. and Carputo, D. 2011. Secondary metabolite profile in induced tetraploids of wild *Solanum commersonii* Dun. Euphytica 8, 2226 – 2237.

Coopoosamy, R. and Naidoo, K. 2011. Assessing the potential of *Tetradenia riparia* in treatment of common skin conditions in rural communities of South Africa. African Journal of Microbiology Research 5, 2942-2945.

De Jesus-Gonzalez, L. and Weathers, P. 2003. Tetraploid *Artemisia annua* hairy roots produce more artemisinin than diploid. Plant Cell Reports 21, 809-813.

Dhawan, O. and Lavania, U. 1996. Enhancing the productivity of secondary metabolites via induced polyploidy: A review. Euphytica 87, 81-89.

Dhooghe, E., Van Laere, K., Eeckhaut, T., Leus, L. and Van Huylenbroeck, J. 2011. Mitotic chromosome doubling of plant tissues *in vitro*. Plant Cell Tissue and Organ Culture 104, 359-373.

Dijkstra, H. and Speckmann, G. 1980. Autotetraploidy in caraway (*Carum carvi* L.) for the increase of the aetheric oil content of the seed. Euphytica 29, 89-96.

du Plooy, W., Regnier, T. and Combrinck, S. 2009. Essential oil amended coatings as alternatives to synthetic fungicides in citrus postharvest management. Postharvest Biology and Technology 53, 117-122.

Gazim, Z., Demarchi, I., Lonardoni, M., Amorim, A., Hovell, A., Rezende, C., Ferreira, G., De Lima, E., De Cosmo, F. and Cortez, D. 2011. Acaricidal activity of the essential oil from *Tetradenia riparia* (Lamiaceae) on the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari; Ixodidae). Experimental Parasitology 129, 175-178.

Griesbach, R. and Kamo, K. 1996. The effect of induced polyploidy on the flavonols of petunia 'Mitchell'. Phytochemistry 42, 361-262.

Hannweg, K., Sippel, A. and Bertling, I. 2013. A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea*. South African Journal of Botany 88, 367-372.

Hannweg, K., Steyn, W. and Bertling, I. 2015. *In vitro*-induced tetraploids of *Plectranthus esculentus* are nematode-tolerant and have enhanced nutritional value. Euphytica, DOI: 10.1007/s10681-015-1547-4.

Janaki Ammal, E. and Sobti, S. 1962. The origin of Jammu Mint. Current Science 31, 387-388.

Klieber, A., Scott, E. and Wuryatmo, E. 2002. Effect of method of application on antifungal efficacy of citral against postharvest spoilage fungi of citrus in culture. Australasian Plant Pathology. 31, 329–332.

Kouassi, K., Bajji, M., Zhiri, A., Lepoivre, P. and Jijakli, M. 2010. Evaluation of three essential oils as potential sources of botanical fungicides. Communications in Agricultural and Applied Biological Sciences 75, 525-529.

Koutoulis, A., Price, A., and Leggett, G. 2005. Polyploid breeding and mutagenesis in Hop (*Humulus lupulus* L.). Acta Horticulturae 668, 41-46.

Lavania, U. 1988. Enhanced productivity of the essential oil in the artificial autopolyploid of vetiver (*Vetiveria zizanioides* L. Nash). Euphytica 38, 271-276.

Lavania, U. 2005. Genomic and ploidy manipulation for enhanced production of phyto-pharmaceuticals. Plant Genetic Resources 3, 170-177.

Levy, M. 1976. Altered glycofavone expression in induced autotetraploids of *Phlox dummondii*. Biochemical Systematics in Ecology 4, 249-259.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Planatrum 15, 473-497.

Ndamane, Y., Kambizi, L., Songca, S. and Oluwafemi, O. 2013. Antibacterial effectiveness of *Tetradenia riparia* extract, a plant traditionally used in the Eastern Cape Province to treat diseases of the respiratory system. Journal of Medicinal Plants Research 7, 2755-2760.

Ochatt, S., Patat-Ochatt, E. and Moessner, A. 2011. Ploidy level determination within the context of *in vitro* breeding. Plant Cell Tissue and Organ Culture 104, 329-341.

Omidbaigi, R., Mirzaee, M., Hassani, M. and Sedghi, M. 2010. Induction and identification of polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment. International Journal of Plant Production 4, 87-98.

Ott, L. and Longnecker, M. 2001. An introduction to statistical methods and data analysis. Australia: Duxbury.

Pattanaik, J., Yogesh, K., Yand, S. and Khatri, R. 2013. *Acorus calamus* Linn.: A herbal tonic for central nervous system. Journal of Scientific and Innovative Research 2, 950-954.

Ranney, T. 2006. Polyploidy: From Evolution to new plant Development. Combined Proceedings of the International Plant Propagator's Society 56, 137 – 142.

Rita, P. and Animesh, D. 2011. An updated overview on Peppermint (*Mentha piperita* L.). International Research Journal of Pharmacy 2, 1-10.

SAS Institute. 2015. The Statistical Procedure manual. North Carolina 27513: SAS Campus Drive, Cary.

Shapiro, S. S., and Wilk, M. B. 1965. An analysis of variance test for normality (complete samples), Biometrika 52, 591–611.

Svehlikova, V. and Repcak, M. 2008. Variation of apigenin quantity in diploid and tetraploid *Chamomill arecutita* (L.) Rausche. Plant Biology 2, 403-407.

Tabassum, N. and Vidyasagar, G. 2013. Antifungal investigations on Plant Essential Oils. A review. International Journal of Pharmacy and Pharmaceutical Sciences 5, Supplement 2, 19-28.

Van Puyvelde, L., Lefebvre, R., Mugabo, P., De Kimpe, N. and Schamp, N. 1987. Active Principles of *Tetradenia riparia*; II. Antispasmodic Activity of 8(14), 15-Sandaracopimaradiene-7α, 18-diol. Planta Medica 53, 156-158.

Van Puyvelde, L., Ntawukiliyayo, J., Portaels, F. and Hakizamungu, E. 1994. In-vivo inhibition of mycobacteria by Rwandese medicinal Plants. Phytotherapy Research 8, 65-69.

Wannakrairoj, S. and Wondyifraw, T. 2013. *In vitro* chromosome doubling in Korarima [*Aframomum corrorima* (Braun) P.C.M. Jansen using colchicine and oryzalin. Kasetsart Journal of Natural Science 47, 684 – 694.

Weaver, D., Dunkel, F., Cusker, J. and Van Puyvelde, L. 1992. Oviposition patterns in two species of bruchids (coleopteran: bruchidae) as influenced by the dried leaves of *Tetradenia riparia*, a perennial mint (lamiales: Lamiaceae) that suppresses population size. Environmental Entomology 21, 1123-1129.

Wohlmuth, H., Leach, D., Smith, M. and Myers, S. 2005. Gingerol content of diploid and tetraploid clones of ginger (*Zingiber officinale* Roscoe). Journal of Agricultural and Food Chemistry 53, 5772-5778.

CHAPTER 5

In vitro-induced tetraploids of *Plectranthus esculentus* are nematode-tolerant and have enhanced nutritional value

K. Hannweg[1,2*], W. Steyn[1] and I. Bertling[2]

[1]Plant Improvement, Agricultural Research Council – Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, 1200, South Africa

[2]Horticultural Science, School of Agricultural, Earth and Environmental Sciences,
Private Bag X01, University of KwaZulu-Natal, Scotsville, 3209

*Corresponding author: K. Hannweg

e-mail: Karin@arc.agric.za
tel: +27 13 753 7000
fax: +27 13 752 3854

Published in Euphytica DOI: 10.1007/s10681-015-1547-4).

Impact factor: 1.385 (2015)

This manuscript is formatted according to the requirements of Euphytica.

In vitro-induced tetraploids of *Plectranthus esculentus* are nematode-tolerant and have enhanced nutritional value

K. Hannweg[1,2*], W. Steyn[1] and I. Bertling[2]

[1]Plant Improvement, Agricultural Research Council – Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, 1200, South Africa

[2]Horticultural Science, School of Agricultural, Earth and Environmental Sciences,
Private Bag X01, University of KwaZulu-Natal, Scotsville, 3209

*Corresponding author: K. Hannweg

e-mail: Karin@arc.agric.za
tel: +27 13 753 7000
fax: +27 13 752 3854

Abstract

Plectranthus esculentus (Family: Lamiaceae), or Livingstone potato, is an edible tuberous vegetable which originated in Africa, with central Africa being the centre of origin. *Plectranthus esculentus* is found throughout the continent, including the north-eastern regions of South Africa. Although the tubers are edible, limited crop improvement has been achieved; therefore, a study comprising *in vitro* polyploidisation was carried out with subsequent evaluation of plant nutritional value and nematode tolerance of the induced tetraploids compared with the diploid controls. Tetraploid tubers had a higher starch content compared with the diploids, however there was no significant difference in mineral element content for either the leaves or the tubers when induced tetraploids were compared with the diploid control. Further, induced tetraploids appeared to be significantly more tolerant to rootknot nematode, *Meloidogyne* spp., than the diploids. A significantly higher number of egg masses per root system and number of eggs and J2 (juvenile stage 2) individuals per root system were detected in control plants, compared with tetraploid plants. Induced tetraploidy resulted in plants with a higher nutritional starch concentration and tolerance to rootknot nematode, characteristics which will improve the cultivation and utilisation of the crop. Morphologically, tetraploid plants had fewer, thicker stems per plant compared with diploid plants.

Keywords

Livingstone potato, crop improvement, chromosome doubling, polyploid

Introduction

Root and tuber crops are globally amongst the most important staple foods. The introduction of crops developed and/or improved using biotechnological methods combined with traditional breeding and selection methods as well as optimum soil fertility and pest management measures could significantly improve yield and crop nutritional value. In general, very little research has been carried out on the nutritional value of edible wild root and tuber species, although indigenous knowledge throughout the world suggests that numerous such species are nutrient-rich and have potential for further development. Moreover, because of their greater tolerance of sub-optimal conditions, the potential of under-utilised wild species to contribute to food security is currently being investigated world-wide. *Plectranthus esculentus,* the Livingstone potato, originated in central Africa, but is distributed throughout the Continent, including the north-eastern regions of South Africa (Codd 1985). The plant is a perennial shrub of 600 mm to 1200 mm in height, with square pubescent stems and subsessile, oppositely-arranged leaves. The edible parts of the plant are the tubers, which are produced by a fibrous root system. The tubers are cylindrical, sometimes branched, 50-100 mm in length and up to 20 mm in diameter. Although there is little information describing cultivation practises, the plant is propagated from sections of stem tubers, usually planted 50 mm to 100 mm deep, on prepared ridges or in beds. After harvesting, tubers used for vegetative propagation can be successfully stored for at least two months under cool, well-ventilated conditions for planting in the following season (Dhliwayo 2002). The species produces tubers even under extremely adverse climatic conditions and therefore contributes to food security in rural areas (Van Wyk and Gericke 2000). Although *P. esculentus* is cultivated throughout Africa as an important vegetable crop, one of the major cultivation challenges experienced by smallholder farmers is a high susceptibility to rootknot nematode (Goodey et al. 1965; Allemann 2002; Xaba and Coertse 2011), made especially more challenging due to the cultivation of other vegetable crops on their lands with consequently high nematode populations in the soil.

A relatively common technique, polyploid induction, has been used in plant breeding since the early 1900's for the development and improvement of a variety of economically-important crops (Gates 1909, Stebbins 1947). Blakeslee and Avery (1937) reported on the first applications in agricultural crops. Such is its importance that a number of reviews have been published in the last decade (Osborn et al. 2003; Soltis et al. 2004; Wendel and Doyle 2005; Chen and Ni 2006; Balao et al. 2011), several highlighting enlargement and induced vigour as well as the enhancement of pest and disease tolerance. Mehta and Swaminathan (1957) and Busey et al. (1993) reported

on the higher resistance of polyploids compared with diploids to nematode parasitism. Although polyploidy is a naturally-occurring phenomenon (Soltis and Soltis 2009; Jiao et al. 2011) it can also be artificially induced. Mitotic spindle inhibitors such as colchicine and oryzalin are most widely used to induce chromosome doubling (Dhooghe et al. 2011). Polyploidy has been induced in several species of the Lamiaceae family including basil (Omidbaigi et al. 2010) and lavender (Urwin 2014). Previous studies in our laboratory, and by others (Beltram and Kam 1984; Ramachandran 1982; Ramachandran and Nair 1992; Smith et al. 2004) showed that various plant characteristics of culinary ginger (a rhizomatous species), *Zingiber officinale*, could be improved by inducing polyploidy. Further, several highly-prized and staple root crop species such as potato, sweet potato, turnip, taro, cassava and yam, are natural or induced polyploids (Hilu 1993). Artificial induction of polyploidy therefore provides a means of potentially improving various characteristics of an under-utilised species such as *Plectranthus esculentus*. The aim of this study was, therefore, to induce polyploidy *in vitro* in *P. esculentus* and subsequently evaluate and compare selected plant characteristics, nematode tolerance, yield and nutritional value of diploid and tetraploid tubers.

Materials and Methods

Micropropagation of shoot cultures of P. esculentus

In vitro shoot cultures were obtained from the Agricultural Research Council's Vegetable and Ornamental Plant Institute, South Africa. Shoots were subcultured on a 4-weekly basis on Murashige and Skoog (1962) nutrient medium with 3 g l^{-1} Gelrite® and 30 g l^{-1} sucrose added (proliferation medium). No growth regulators were added to the culture medium. All media was autoclaved for 20 minutes at 121 °C and 1KPa. Shoot cultures were grown at 25 to 27 °C with cool white fluorescent light (81 µmol m−2 s−1, Phillips 65 W) 27 under a 16:8 hour light:dark cycle and shoots rooted spontaneously. Rooted plantlets were washed under running tap water before being planted into Speedling® trays for acclimatisation in a mist-bed at 85% shading and misting for 5 seconds every hour for 3 weeks, before being transplanted into larger planting bags containing a 1:1 [v/v] mixture of sand and pine bark. Plants were maintained in a 40% black shade cloth shadehouse.

Induction of polyploidy

Single nodes were excised from *in vitro* shoot cultures and subsequently incubated in sterile (autoclaved) solutions containing 0.01 g l^{-1}, 0.10 g l^{-1}, 1.0 g l^{-1} and 10.0 g l^{-1} colchicine, either overnight or for three days. Two hundred and fifty nodes were used for each treatment. The treated nodes were then cultured on proliferation medium, allowing the axillary buds to grow out and elongate. Once shoots were large enough to be harvested for flow cytometry analysis, usually after 3-4 weeks, samples were collected to verify polyploid induction.

Verification of polyploid induction

Ploidy level of shoots resulting from colchicine treatment was verified using flow cytometry. Samples were prepared for flow cytometry analysis using approximately 0.5 cm^2 leaf tissue. The tissue was macerated with a razor blade in 125 µl nucleus extraction solution (Partec, Germany), after which the homogenate was filtered through a 50 µm mesh filter. The isolated nuclei were stained with 1250 µl of 4'-6-diamidino-2-phenylindole (DAPI) stain (Partec, Germany) prior to commencing flow cytometry analysis using a Partec CYFLOW space flow cytometer. Nuclei isolated from untreated, diploid plantlets were used as a standard. Histograms were analysed using the Partec software package. Shoots confirmed as tetraploid were proliferated on MS medium under the conditions described above. Plantlets were acclimatised as described above. After 12 weeks, plants were transplanted into 20 l potting bags containing a 1:1 [v/v] mixture of pine bark and sand and controlled-release fertiliser granules (Osmocote®).

Morphological characterisation

Morphological characteristics such as plant growth form, number of shoots produced per plant and leaf shape were evaluated and compared between diploid and tetraploid plants.

Nutritional analysis of tubers and leaves

Mature, but not senescing, leaves were harvested from plants and were analysed for macro- and micronutrient content. Tubers were harvested eight months after the acclimatised plantlets were planted into the 20 l potting

bags. All analyses were carried out using standard methods (at a South African National Accreditation System-accredited facility) particular to each nutrient with three replicates, twelve plants per replicate for each analysis for both diploids and tetraploids.

Leaf samples were oven-dried at 60 ºC for 48 hours before being finely milled. Milled samples were extracted overnight using 2:1 nitric acid (55%), [v/v]: perchloric acid (70%) solution. Samples were then digested for 6 hours at 180 ºC on a digestion block before being cooled. Calcium, magnesium, zinc, copper, manganese and iron were determined using atomic absorption spectrophotometry (AOAC Official Method 975.03) whereas potassium and sodium were analysed using flame emission spectroscopy (Varian SpectraAA 250 Plus) according to the method described by Poluektov (1973). Phosphorous and boron were analysed colorimetrically, phosphorous at 660 nm and boron at 430 nm, using an Auto Analyzer (Bran and Luebbe Auto Analyser 3) according to standard methods, Phosphorus reagents, Technicon Industrial Method, method 144-71A (1972) and AOAC Official Method 982.01, respectively.

For nitrogen analysis, samples were oven-dried at 60 ºC for 48 hours before being extracted for 2 hours with concentrated sulphuric acid followed by digestion with hydrogen peroxide. Nitrogen was analysed colorimetrically at 640 nm (ammonia-salicylate complex) using an Auto Analyzer (Bran and Luebbe Auto Analyser 3 (AOAC Official Method 990.02).

The β-carotene content of tubers was analysed using standard methods as described by Horwitz (2000) by the South African Bureau of Standards, Pretoria, South Africa.

Diploid and tetraploid tuber material was analysed for ash, protein, carbohydrate and fat content as well as for vitamins B1 and B2. Amino acid content (serine, aspartic acid, glutamic acid, glycine, threonine, alanine, tyrosine, proline, methionine, valine, phenylalanine, isoleucine, leucine, histidine and lycine) was also determined according to Gehrke et al. (1985).

Ash content was determined by heating the sample at 550 ºC overnight. The remaining residue (inorganic matter) was used to determine ash content (AOAC Official Methods 934.01 and 930.15). Protein content of samples was determined using the Kjeldahl method which measures total organic nitrogen (AOAC Official Method 954.01). The organic matter was digested with hot concentrated sulphuric acid and a catalyst mixture added to the acid to raise the boiling point. All nitrogen was converted to ammonia which was measured by titration. Soluble and insoluble carbohydrates were determined by analysing moisture content, crude protein, ash and fat content with

the remainder being total carbohydrates (Greenfield and Southgate 2003). Soluble unbound fat was dissolved in ether at boiling point and evaporated at 105 ºC using the Soxtec method (AOAC Official Method 920.39 and its content expressed as a percentage.

The tissue vitamin B1 concentration was determined by derivatising samples to form thiochrome (a highly fluorescent oxidised product of thiamine) after autoclave extraction. A C18 cartridge was used to remove interferences and the vitamins chromatographed by using reversed phase separation as described by Sims and Schoemaker (1993). The vitamin B2 concentration was determined after autoclave extraction, centrifugation and dilution. The samples were analysed using reversed phase separation.

Amino acid content was analysed as described by Einarsson et al. (1983) using acid hydrolysis followed by pre-column derivatisation, HPLC separation and detection using a fluorescence detector.

Starch was determined using an iodine-based colorimetric method as described by Xu et al. (1998). Tubers were oven-dried at 65 ºC for 48 hours for sample preparation and starch content was determined at 620nm using a WPA-lightwave spectrophotometer.

Nematode studies

Fifteen plants each of diploid control plants and induced tetraploids were evaluated for their tolerance to local *Meloidogyne incognita* race 2 and *M. javanica* populations in a greenhouse experiment. To test the virulence of the nematodes, a highly susceptible tomato (*Solanum lycopersicon* L.) cv. 'Rodade' was used as a reference plant in all experiments. One litre (1l) black plastic potting bags were filled with steam-pasteurised sandy soil (84% sand, 14% silt, 2% clay and 0.5% organic matter content). The soil pH (H_2O) was 5.75. Nutrition in the form of Multifeed® Classic (Efekto), was applied as a soil drench every 14 days at a rate of 7.5g l^{-1} water. One plant was planted per bag. Populations of *M. incognita* race 2 and *M. javanica* were established and maintained on the 'Rodade' tomatoes in a separate greenhouse. Eggs and second stage juveniles (J2) of each of the appropriate species were used to inoculate plants. Inoculation was performed 32 days after planting by pipetting approximately 1000 eggs and J2 of the respective population on exposed roots of each of the seedlings. The roots were covered with soil again after the inoculation.

Fifty six days after nematode inoculation, the plants were carefully removed from the bags. This period allowed completion of at least one nematode generation (Kleynhans 1991; Fourie 2005). The root systems were rinsed free of adhering soil and debris with running tap water and blotted dry on paper towel. The number of egg masses

per root system was counted. Staining of the egg masses to facilitate counting was done by immersion of the root systems in a Phloxine B solution for 20 minutes (Hussey and Boerma 1981). Root systems were individually inspected and the red-stained egg masses were counted using a stereo microscope. Eggs and J2 were extracted from the root systems using Riekert's (1995) modified NaOCl method. Eggs and J2 were counted and the reproductive potential of each nematode population on each genotype was determined according to Oosterbrink's reproduction factor (Windham and Williams 1988) and calculated as follows: Rf = final egg and J2 numbers (Pf) / initial egg and J2 numbers (Pi).

Statistical Analysis

For the polyploid induction/colchicine applications, an appropriate analysis of variance was fitted to the data using PROC GLM procedure of SAS software Version 9.2 of the SAS System for Windows (SAS Institute, 2015). Shapiro-Wilk test was performed to test for normality (Shapiro and Wilk 1965) and a Fisher t-test with Least Significant Difference was calculated at the 5% significance level to compare treatment means (Ott and Longnecker, 2001). For all other experiments, the experimental layout was a complete randomised design (CRD) with 3 replications, twelve measurements per replicate. An analysis of variance was performed at the 5% significance level using SAS software Version 9.2 of the SAS System for Windows (SAS Institute, 2015).

Results and Discussion

In vitro proliferation and acclimatisation of P. esculentus shoots

Plectranthus esculentus shoots were readily proliferated and spontaneously rooted *in vitro*. There was no difference between the multiplication rate (5.0 at a subculture interval of 6 weeks) of diploid and tetraploid plants, although the tetraploid plants did have a tendency for thicker stems and larger leaves *in vitro* than the diploids. Both diploid and tetraploid plants were readily acclimatised in the mistbed using the methods described, and transplanted into 1 l potting bags 3-4 weeks after acclimatisation.

Verification of polyploid induction of in vitro *shoot cultures P. esculentus using flow cytometry analysis*

Flow cytometry analysis facilitated the identification of pure polyploid plants from the colchicine-treated population containing diploids, mixoploids and polyploids. Polyploidy was induced in *in vitro* shoots of *Plectranthus esculentus* for all treatments except the three day 10 g l^{-1} colchicine treatment where all shoots died. A relatively high percentage of tetraploids was induced and survived for the overnight, 1.0 g l^{-1} (20.40%), three day, 0.1 g l^{-1} (11.20%) and three day, 1.0 g l^{-1} (26.80%) treatments (Table 1). No octoploids were identified. The interaction of exposure time and colchicine concentration (p-value = 0.5501) as well as exposure time and ploidy interaction (p-value = 0.9987) were not significant. Further, an investigation of comparison of means for the interaction between concentration and ploidy level was also not significant. However treatment of nodes at 10 g l^{-1} at both exposure times tested appeared to be toxic based on the extremely high mortality of shoots. Earlier studies reported that successful chromosome doubling is dependent on explant type, duration of exposure and concentration of colchicine as well as the genome doubling capacity of the species under investigation (Khosravi et al. 2008; Sun et al. 2009; Dhooghe et al. 2011) and therefore each species requires testing.

Table 1 Effect of colchicine exposure time and concentration on polyploidy induction of *in vitro* shoots of *Plectranthus esculentus*. Data is expressed based on the number of nodes surviving as a percentage of the total number of nodes treated for each exposure time and concentration.

Treatment (g/l)		2n (%)	2n/4n (%)	4n (%)	4n/8n (%)	8n (%)
Overnight	0.00	100.0	0.00	0.00	0.00	0.00
	0.01	29.60	0.00	2.40	0.00	0.00
	0.1	54.80	0.00	7.60	0.00	0.00
	1.0	42.00	0.00	20.40	0.00	0.00
	10.0	2.80	1.20	0.40	0.00	0.00
3 days	0.00	100.0	0.00	0.00	0.00	0.00
	0.01	90.40	4.80	1.60	0.00	0.00
	0.1	8.80	8.80	11.2	4.0	0.00
	1.0	26.80	0.00	26.80	0.00	0.00
	10.0	0.0	0.0	0.0	0.0	0.0

Morphological characteristics of polyploid P. esculentus plants

In contrast to diploids, induced tetraploid plants showed marked differences amongst various morphological characteristics compared with the diploids (Table 2). Tetraploid plants had, on average, fewer stems than their diploid progenitors (11 stems per plant compared with 24 stems per plant, respectively). Furthermore, stem diameter, was also significantly larger for the induced tetraploids compared with the diploids (5.30 mm compared with 3.29 mm, respectively), although there was no difference in plant height. Leaf index, an indication of leaf shape, was higher for the tetraploids than the diploids, but it was not significant. Leaves of the tetraploid plants were a darker shade of green than the diploids. The alteration of leaf morphology of *P. esculentus* is in line with reports that polyploidisation can alter plant morphology, phenology and physiology (Levin 2002). In a review of polyploidy in plants, Tate et al. (2005) reported on a variety of well-documented studies in which ploidy level alterations can result in higher growth rates, increased secondary metabolite production as well as larger vegetative and reproductive plant parts – particularly in ornamental and food crops.

Table 2 Comparison of various plant characteristics between diploid and tetraploid *Plectranthus esculentus* plants. n = 12 each for diploid and tetraploid plants, 3 replicates, $P<0.05$). Different letters within columns represent significant differences.

	Number of stems/plant	Stem diameter (mm)	Leaf index (breadth/length)
diploid	24.0b	3.29a	0.41a
tetraploid	11.0a	5.30b	0.51b

Nutritional characteristics of polyploid P. esculentus plants

Tuberous crops are cultivated for their starch-rich storage organs which are a rich source of energy in the form of carbohydrates, including starch. There is a dearth of literature describing the impact of induced polyploidy on starch content of tubers. However, most commercial tuber crops such as potato, sweet potato, yam and cassava are all polyploid, many of them being high-yielding triploid varieties (Atherton and Rees 2008) compared with the diploids. Although dry matter content, a characteristic important in the improvement of tuber crops, was not significantly different between the diploid and tetraploid plants (results not shown), tuber starch content of the tetraploid plants was significantly higher than that of the diploids (47.1% on a dry weight basis compared with 40.0% for the diploids). Although these figures are lower than that reported previously for *P. esculentus* (Temple et al. 1991), this difference may be ascribed to factors such as cultivation, edaphic conditions or analytical procedures. The starch content still remains significantly higher than potato, 17.1%, (Potato Board, 1980) and sweet potato, average of 23%, (ARC, 1979). β-Carotene, an important anti-oxidant in tuber crops and precursor of provitamin A, while higher for the tetraploids than the diploids, it was not significantly so, 0.112 mg kg^{-1} compared with 0.07 mg kg^{-1} respectively, under the cultivation conditions of the experimental trial (Table 3). Vitamin A is an essential vitamin and is required for the development and maintenance of eyesight. An extreme deficiency can ultimately lead to the development of blindness. Vitamin A is also required by the body for healing processes after wounding or infection. Polyploidy affects the biosynthesis of a number of metabolites of a variety of biochemical pathways in plants and are generally present in higher concentrations in polyploids than diploids. Several studies involving the mechanism/s thereof have been carried out (Dhawan and Lavania 1996; Zhang et al. 2005; Caruso et al. 2011) although there are few studies describing the effect of polyploidy on β-carotene biosynthesis and levels in induced polyploids. Jaskani et al. (2005), however, reported that β-carotene content was higher in induced tetraploids of watermelon compared with the diploids. There were no significant differences between diploids and tetraploids in terms of any of the other macro- and micronutrients evaluated (Table 4), but this, together with the effect of polyploidy on β-carotene, needs to be investigated further under in-field cultivation conditions which are sub-optimal compared with a pot trial.

Table 3 Comparison between selected nutritional values of tubers harvested from diploid and tetraploid *P. esculentus* plants. n = 12 each for diploid and tetraploid plants, 3 replicates, $P<0.05$). The same letter across rows indicates no significant difference. (results are expressed as fresh weight)

Component	Diploid	Tetraploid
Ash (%)	0.64a	0.61a
Protein (%)	0.82a	0.79a
Carbohydrates (%)	21.78a	18.99a
Fat (%)	0.198a	0.268a
Starch (g/kg)	400a	470b
β-Carotene (mg/kg)	0.07a	0.112a
Vitamin B1 (mg/100g)	0.01a	0.01a
Vitamin B2 (mg/100g)	0.01a	0.01a
Alanine (g/100g)	0.042a	0.036a
Aspartic acid (g/100g)	0.056a	0.050a
Glutamic acid (g/100g)	0.07a	0.06a
Histidine (g/100g)	0.04a	0.056a
Isoleucine (g/100g)	0.032a	0.032a
Leucine (g/100g)	0.034a	0.032a
Lysine (g/100g)	0.038a	0.034a
Methionine (g/100g)	0.01a	0.01a
Phenylalanine (g/100g)	0.036a	0.032a
Proline (g/100g)	0.028a	0.022a
Serine (g/100g)	0.034a	0.032a
Threonine (g/100g)	0.046a	0.042a
Tyrosine (g/100g)	0.022a	0.028a
Valine (g/100g)	0.038a	0.036a

Table 4 Comparison between selected mineral values of leaves and tubers harvested from diploid and tetraploid *P. esculentus* plants. n = 12 each for diploid and tetraploid plants, 3 replicates, P<0.05). The same letter across rows indicates no significant difference. Results are presented as per dry mass.

Component	diploid	tetraploid
	LEAVES	
N (%)	2.65a	3.10a
P (%)	0.154a	0.153a
K (%)	2.01a	2.23a
Ca (%)	1.11a	1.00a
Mg (%)	0.554a	0.546a
Zn (mg/kg)	46.75a	41.75a
Cu (mg/kg)	9.00a	8.33a
Mn (mg/kg)	804a	714.14a
Fe (mg/kg)	94.91a	91.63a
B (mg/kg)	32.5a	19.7a
	TUBERS	
N (%)	0.59a	0.63a
P (%)	0.122a	0.125a
K (%)	1.22a	1.14a
Ca (%)	0.10a	0.08a
Mg (%)	0.149a	0.141a
Zn (mg/kg)	17.00a	17.40a
Cu (mg/kg)	3.40a	4.00a
Mn (mg/kg)	16.60a	17.5a
Fe (mg/kg)	34.50a	34.75a
B (mg/kg)	17.40a	18.8a
Na (%)	0.0238a	0.0212a

Tolerance of polyploid plants to nematodes

One of the most significant biotic factors affecting any tuber crop is nematode infestation. Nematodes cause significant yield losses and applications of both chemical and environmentally-friendly products to reduce the risk of damage to crops is an industry worth millions of US dollars. Management strategies, as well as breeding and selection of more tolerant varieties, is critical for any tuber crop industry (Jones et al. 2011). *Plectranthus esculentus* is highly susceptible to rootknot nematodes (*Meloidogyne* spp.). Tetraploid and diploid plants were challenged with rootknot nematodes in a pot experiment to determine if polyploidy resulted in any degree of tolerance. The results of the trial showed that tetraploid plants were significantly more tolerant to rootknot nematodes (Table 5). There was a significant difference in the number of egg masses per root system for the diploids compared with the tetraploids. Tetraploid plants had a significantly lower egg mass number per root system compared with the diploids (7.9 egg masses/root system compared with 28 egg masses/root system,

respectively). Tetraploids were therefore found to be moderately resistant to rootknot nematodes compared with the diploids which were highly susceptible according to the classification system of Murray et al. (1986). Similarly, the number of eggs and J2 per root system was also significantly lower for the tetraploids compared with the diploids, 3196 compared with 12187, respectively). The reproduction factor (Rf-value) is an indication of tolerance to nematode infestation, the lower the Rf-value the more tolerant the plant species is to nematode attack. Tetraploids had a significantly lower Rf-value, 3.17, compared with the diploids, 12.15. Although there was a significant improvement in nematode tolerance over the diploids, host status is considered good compared with diploids with a host status defined as excellent according to the Rf classification system described by Windham and Williams (1988). In this study, the tolerance of tetraploids compared with diploids to rootknot nematode was investigated and examination of tuber anatomy at light microscope level did not reveal any fundamental differences between the induced tetraploids and diploids prior to nematode challenging, nor after challenging, possibly due to the young plant age. However, future histopathological studies may reveal symptoms of nematode infestation such as feeding sites and associated changes in cell structure on more established plants. In potato, rootknot nematode damage to tubers which is characterised not only by losses in yield, but damage can manifest as blistering on the surface of tubers as well as internal browning (Vovlas et al. 2005). Furthermore, Jatala et al. (1982) reported that externally-visible symptoms may only develop after a period of storage after internal symptoms have developed on potato. This manifestation of tuber damage is critical in terms of *P. esculentus* tubers which are commonly stored for relatively long periods of time before consumption or re-establishment (van Wyk and Gericke 2000) and this should be investigated further. Moreover, there is the possibility that growers unwittingly facilitate the spread of nematodes using diseased seed tubers which may not yet exhibit symptoms externally.

Table 5 Comparison of number of egg masses per root system, number of eggs and J2 per root system and reproduction factor value for diploid and tetraploid *Plectranthus esculentus* plants inoculated with J2 and eggs of *Meloidogyne* species. n = 15 each for diploid and tetraploid plants, 3 replicates ($P<0.05$). Different letters within columns represent significant differences.

	Number of egg masses per root system	Number of eggs and J2 per root system	Reproduction factor value (Rf-value)
Diploid	28b	12187b	12.15b
Tetraploid	7.9a	3196a	3.17a

Future prospects regarding improvement

Almost no breeding and selection or improvement has been carried out on *Plectranthus esculentus*. This is the first report on the improvement of this species using artificial polyploid induction. Furthermore, it is also one of the few published reports on the induction of nematode tolerance in induced polyploid plants. Although the histopathological response/s and manifestation of symptoms of infestation as well as the mechanism of tolerance to nematodes by the tetraploid plants still needs to be elucidated, polyploid induction could provide a valuable tool in improving nematode-tolerance in a number of crops; however, the effect of polyploidy on other critical horticultural characteristics such as yield and crop quality would need to be taken into account and investigated. Physiological investigations i.e. assimilation and water stress studies are being explored further after preliminary investigations revealed that the tetraploids appeared to have higher assimilation rates and superior water stress tolerance than the diploids.

Acknowledgements

The authors would like to thank the Agricultural Research Council and Department of Science and Technology (ECS Programme) for financial assistance. Mr Gerrit Visser and Ms Elsabe Aylward of the ARC-Institute for Tropical and Subtropical Crops are thanked for technical assistance for the flow cytometry and mineral nutrition samples, respectively, as well as ARC-Irene Analytical Services for the nutritional analyses. Mardé Booyse of the ARC Biometry Unit is thanked for assistance with the statistical analysis.

References

Alleman J (2002) Evaluation of *Plectranthus esculentus* as a potential vegetable crop. Ph.D Dissertation, University of Pretoria, South Africa.

ARC (1979) Annual sweet potato research report. ARC-Roodeplaat Vegetable and Ornamental Plant Institute, Pretoria.

Atherton J, Rees A Eds (2008) Tropical Root and Tuber Crops: Cassava, Sweet potato, Yams and Aroids. Crop Protection Science in Horticulture 17, CAB International, Wallingford, United Kingdom.

Balao F, Herrera J, Talavera S (2011) Phenotypic consequences of polyploidy and genome size at the microevolutionary scale: a multivariate morphological approach. New Phyt 192:256-265.

Beltram I, Kam Y (1984) Cyto taxonomic studies in the Zingiberiaceae. Notes from the Royal Bot. Garden, Edinburgh 41:541-557.

Blakeslee A, Avery A (1937) Colchicine and double diploids. J Hered 28:411-412

Busey P, Giblin-Davis R, Center B (1993) Resistance in Stenotaphrum to the sting nematode. Crop Science 33:1066-1070.

Caruso I, Lepore L, de Tommasi N, dal Piaz F, Frusciante L, Aversan R, Garramone R, Carputo D (2011) Secondary metabolite profile in induced tetraploids of wild *Solanum commersonii* Dun. Euphytica 8:2226 – 2237.

Chen Z, Ni Z (2006) Mechanisms of genomic arrangements and gene expression changes in plant polyploids. Bioessays 28:240-252.

Codd L (1985) The genus *Plectranthus*. Flora of S Afr 28:137–172.

Dhawan O, Lavania U (1996) Enhancing the productivity of secondary metabolites via induced polyploidy: a review. Euphytica 87:81-89.

Dhliwayo P (2002) Underexploited tuber crops in Zimbabwe: a study on the production of Livingstone Potato (*Plectranthus esculentus*). PGR Newsletter, FAO-Bioversity 130:77-80.

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissues in vitro. Plant Cell Tiss Org Cult 104:359-373

Einarsson S, Josefsson B, Lagerkvist S (1983) Determination of Amino Acids with 9-Fluorenylmethyl Chloroformate and reversed-phase High-Performance Liquid Chromatography. J Chromat 282:609-618.

Fourie H (2005) *In vivo* evaluation of resistance to *Meloidogyne incognita* race 2 (Nematoda: Tylenchida) and identification of genetic markers for this trait in soybean (Glycine max). Ph.D. Thesis. Catholic University of Leuven, Belgium.

Gates R (1909) The stature and chromosomes of *Oenanthera gigas* De Vries. Arch für Zellf 3:525-552.

Gehrke C, Wall L, Absheer J, Kaiser, F, Zumwalt, R (1985) Sample preparation for chromatography of amino acids: Acid hydrolysis of Proteins. J Assoc Offic Analyt Chem 68:811-821.

Goodey J, Franklin M, Hooper D (1965) The Nematode Parasites of Plants Catalogued Under Their Hosts. Commonwealth Agricultural Bureaux, Farnham Royal: Bucks, England. 214pp.

Greenfield H, Southgate D (2003) Food composition data – Production, Management and Use. 2nd ed. Food and Agricultural Organization of the United Nations, Rome.

Hilu K (1993) Polyploidy and the evolution of domesticated plants. Am J Bot 80:1494-1499.

Horwitz W (2000) Official methods of analysis of AOAC International, 17th Edition. Gaithersburg.

Hussey R, Boerma H (1981) A greenhouse screening procedure for root-knot nematode resistance in soybeans. Crop Sci 21:794-796.

Jaskani M, Kwon S, Kim D (2005) Comparative study on vegetative, reproductive and qualitative traits of seven diploid and tetraploid watermelon lines. Euphytica 145:259-268.

Jatala P, Booth R, Wiersema S (1982) Development of *Meloidogyne incognita* in stored potato tubers. J Nemat. 14:142–3.

Jiao Y, Wickett N, Ayyampalayam S, Chanderbali A, Landherr L, Ralph P, Tomsho L, Hu Y, Liang H, Soltis P, Soltis D, Clifton S, Schlarbaum S, Schuster S, Ma H, Leebens-Mack J, dePamphilis C (2011) Ancestral polyploidy in seed plants and angiosperms. Nature 473:97-100.

Jones J, Gheysen G, Fenool C Eds. (2011) Genomics and Molecular Genetics of Plant-Nematode Interactions. Chapter 2: 21-43. Springer Science+Business Media B.V.

Kleynhans K (1991) The root-knot nematodes of South Africa. Technical Communication No. 231. Pretoria: Department of Agricultural Development, 61pp.

Khosravi P, Jafarkhani Kermani M, Nematzade G, Bihamta M (2008) Role of mitotic inhibitors and genotype on chromosome doubling of Rosa. Euphytica 160:267-275.

Levin D (2002) The role of chromosomal change in plant evolution. Oxford: Oxford University Press.

Mehta R, Swaminathan M (1957) Studies on induced polyploidy in forage crops. Indian Journal of Genetics and Plant Breeding 17:27-57.

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant 15:473–497.

Murray J, Poole T, Ostazeski, S (1986) Techniques for determining reproduction of *Meloidogyne gramnis* on Zoysiagrass and Bermudagrass. Plant Disease 70:559-560.

Omidbaigi R, Mirzaee M, Hassani M, Sedghi Moghadam M (2010) Induction and identification of polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment. Int J of Plant Prod 4:87-98

Osborn T, Chris Pires J, Birchler J, Auger D, Jeffery Chen Z, Lee H-S, Comai L, Mablung A, Doerge R, Colot V, Martienssen R (2003) Understanding mechanisms of novel gene expression in polyploids. Trend Genet 19:141-147.

Ott R, Longnecker M (2001) An Introduction to Statistical methods and data analysis. 5th Edition Belmont, California: Duxbury Press: p 440 (pp 1-1152) t test and statistical principals.

Poluektov N (1973) Analytical Methods of Flame Photometry. Technika knjiga, Belagrade.

Potato Board (1980) Nutritive value of potatoes. Fmg S Afr Potatoes A2.

Ramachandran K (1982) Polyploidy induced in ginger by colchicine treatment. Curr Sci 51:288-289.

Ramachandran K, Nair P (1992) Cytological studies on diploid and autotetraploid ginger (*Zingiber officinale* Rosc.). J Spices Arom Crops 1:125-130.

Riekert F (1995) An adapted method for extraction of root-knot nematodes from maize root samples. Afr. Plant Prot 1:41-43.

SAS Institute (2015) The Statistical Procedure manual. North Carolina 27513: SAS Campus Drive, Cary.

Shapiro, S, Wilk, M (1965) An Analysis of Variance Test for Normality (complete samples). Biometrika 52: 591-611.

Sims A, Shoemaker D (1993) Simultaneous Liquid Chromatographic Determination of Thiamine and Riboflavin in selected Foods. J AOAC Intl 76:1156-1160.

Smith M, Hamill S, Gogel B, Severn-Ellis A (2004) Ginger (*Zingiber officinale*) autotetraploids with improved processing quality produced by an in vitro colchicine treatment. Austr J Exp Agric 44:1065–1072.

Soltis D, Soltis P and Tate J (2004) Advances in the study of polyploidy since plant speciation. New Phyt 161:173-191.

Soltis P, Soltis D (2009) The Role of Hybridization in Plant Speciation. Annu Rev of Plant Biol 60:561-588.

Stebbins G (1947) Types of polyploidy: their classification and significance. Adv Genet 1:403-429.

Sun Q, Sun S, Li L, Bell R (2009) *In vitro* colchicine-induced polyploidy plantlet production and regeneration from leaf explants of the diploid pear (*Pyrus communis* L.) cultivar 'Fertility'. J Hortic Sci Biotech 84:548–552.

Tate J, Soltis D, Soltis P (2005) Polyploidy in Plants. In The Evolution of the Genome, Elsevier Inc. pg. 371-426.

Temple VJ, Ojobe TO, Onobun CE (1991) Chemical composition of Livingstone potato tubers (*Plectranthus esculentus*). J Sci Food Agric 56:215-217.

Urwin N (2014) Generation and characterisation of colchicine-induced polyploid *Lavandula* x *intermedia*. Euphytica 197:331-339.

Van Wyk B-E, Gericke N (2000) People's Plants: a guide to useful plants of southern Africa. (351 pages). Briza Publications, Pretoria.

Volvlas N, Mifsud D, Landa B, Castillo P (2005) Pathogenicity of the root-knot nematode *Meloidogyne javanica* on potato. Plant Path. 54:657-664.

Wendel J, Doyle J (2005) Polyploidy and evolution in plants. In R Henry (ed.) Plant diversity and evolution: Genotypic and phenotypic variation in higher plants. CAB International.

Windham G, Williams W (1988) Reproduction of *Meloidogyne javanica* on corn hybrids and Inbreds. Ann App Nemat 2:25-28.

Xaba P, Croeser P (2011) Wild Potato. Veld and Flora, December 2011:180-181.

Xu C, Chen, W Chen, K, Zhang, S (1998) A simple method for determining the content of starch–iodine colorimetry. Biotech 8:41-43.

Zhang A, Chang L, Xue J (2005) Progress in research on inducing the polyploid of medicinal plants. Zhongguo Zhong Yao Za Zhi. 30:645-9. Review. Chinese

CHAPTER 6

Micropropagation and *in vitro* Polyploidisation of *Siphonochilus Aethiopicus* (Wild Ginger) and its Effect on Selected Horticultural Characteristics

K. Hannweg[1,2,a], G. Visser[1], A . Sippel[1] and I. Bertling[2]

[1]Agricultural Research Council, Institute for Tropical and Subtropical Crops, Nelspruit, South Africa; [2]School of Agricultural, Earth and Environmental Sciences University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Submitted and accepted for publication in Acta Horticulturae, International Horticulture Congress 2014. To be published in Acta Horticulturae – IHC Symposium 22 (Micropropagation) 2015/2016.

This manuscript is formatted according to the requirements of Acta Horticulturae.

Micropropagation and in vitro polyploidisation of *Siphonochilus aethiopicus* (Wild Ginger) and its effect on selected horticultural characteristics

K. Hannweg[1,2,a], G. Visser[1], A . Sippel[1] and I. Bertling[2]

[1]Agricultural Research Council, Institute for Tropical and Subtropical Crops, Nelspruit, South Africa; [2]School of Agricultural, Earth and Environmental Sciences University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Abstract

***Siphonochilus aethiopicus* (wild ginger) is one of only several thousand plant species used in traditional medicinal preparations in South Africa. The plant is threatened with extinction and is already extinct in the wild in the KwaZulu-Natal Province and is increasingly threatened in the Mpumalanga Province where small populations are reported to exist. The cone-shaped rhizomes and fleshy roots are extremely popular and are widely used in traditional medicines which include treatments for asthma, hysteria, colds, coughs and flu, as well as malaria, amongst others. Due to the unsustainable wild harvesting of this species, efficient in vitro micropropagation and acclimatization protocols were developed in efforts to conserve the species. Furthermore, using the aforementioned protocols, induced polyploid selections were developed in vitro and verified using flow cytometry. The effect of polyploidy on wild ginger with respect to selected horticultural characteristics was evaluated.**

Keywords: *Siphonochilus aethiopicus*, tetraploidy, horticultural characteristics, micropropagation

INTRODUCTION

Over 4,000 plant species are used in traditional medicines throughout southern Africa. Uses are as diverse as the ailments being treated and *Siphonchilus aethiopicus* is no exception. The plant is highly prized for its medicinal value and as a result has been over harvested from the wild almost to extinction and consequently is currently listed in the Red Data book of South African plants. Although the species was widespread, it is now thought to be extinct in the wild in KwaZulu-Natal and only small populations are reported to exist in Mpumalanga (van Wyk and Gericke, 2000). *Siphonochilus aethiopicus*, or wild ginger, (Family Zingiberiaceae) has aromatic rhizomatous roots and grows on forest floors. The leaves are deciduous and sprout annually from the underground stems in spring and plants reach a height of 500 mm to 1 m. The leaves are lance-shaped and borne on the end of stem-like leaf bases. Small berry-like fruits are produced at or near ground level after the flowers (October to February) which are extremely short-lived. The large flowers are extremely delicate and may vary in colour from bright pink to white with a yellow centre and are delicately scented. The cone-shaped rhizomes and fleshy roots are sold on the traditional medicine (muti) markets in South Africa. The highly aromatic roots have a variety of medicinal and traditional uses. It is used by the Zulu people as a protection against lightning

[a]E-mail: Karin@arc.agric.za

and snakes. The rhizomes and roots are chewed fresh to treat asthma, hysteria, colds, coughs & flu. A preparation is administered to horses as prevention against horse sickness. Wild ginger is used by the siSwati people to treat malaria and is chewed by women during menstruation (Hutchings, 1996).

Improvement of selections in terms of yield is an important factor in terms of the survival of the species as well as sustainability of harvesting. Polyploidy, or chromosome doubling, has been reported to result in an increase in not only crop yields but also increases in size and function of plant parts such as flowers, roots, tubers, leaves as well as photosynthesis and secondary metabolite production. In previous experiments carried out in our own laboratories as well as others (Smith et al., 2004; Kun-Hua et al., 2011) on induced tetraploids of culinary ginger, *Zingiber officinale.* Substantial increases in leaf length and width and tuber and flower size were recorded. It was with these results in mind that we investigated the effect of polyploidy on wild ginger with regard to plant morphology, biomass and potential yield.

METHODS AND MATERIALS

Establishment of in vitro cultures of *Siphonochilus aethiopicus*

S. aethiopicus rhizomes were sterilised using various sterilising agents and time intervals as follows. Rhizomes were initially rinsed until all visible soil was removed, 5 min before being placed into a solution of 2 g L^{-1} CAPTAB (active ingredient: captab at 500 g kg^{-1}; Villa Crop Protection) fungicide for 2 h. Thereafter, rhizomes were treated with a 1% [v/v] solution of calcium hypochlorite (CaOCl, 680 g kg^{-1}) containing a few drops of detergent for 15, 20 or 30 min before being rinsed 3 times with sterile distilled water. Rhizome buds were excised from the rhizomes using a sharp scalpel blade and cultured individually in culture tubes containing the MS (Murashige and Skoog, 1962) nutrients and vitamins, 30 g L^{-1} sucrose and solidified with 3 g L^{-1} Gelrite. Buds which did not show any contamination by bacteria and/or fungi were transferred to fresh nutrient medium supplemented with 1 mg L^{-1} benzylaminopurine (BAP) for proliferation. Shoots were then cultured on various combinations of cytokinins and auxins in efforts to stimulate increased rates of shoot multiplication. Single shoots were cultured on medium containing either 1 mg L^{-1}, 2 mg L^{-1} or 4 mg L^{-1} BAP or Kinetin (KIN), alone or in combination with 1 mg L^{-1} indoleacetic acid (IAA). Multiplication rates were assessed over a period of 12 months with explants being subcultured every 4 weeks. To further stimulate shoot proliferation, shoots were longitudinally sliced and cultured on medium supplemented with 2 mg L^{-1} BAP (Smith et al., 2004). The proliferation rate of these 'half' shoots was compared with that of the whole shoots.

Acclimatization of in vitro shoots

Tissue cultured plants of a range of sizes, with (average 6mm shoot base diameter and 30 mm shoot height) and without roots (average 4.5 mm rhizome diameter & 30mm shoot height), were planted into seedling trays containing a 1:1 mixture of pine bark and coarse sand. Before planting, plantlet height and rhizome diameter were measured before being dipped in captab fungicide. The trays were placed in the mist bed for 4 weeks before the plants were transferred to individual potting bags. Shoot growth rate, root length, rhizome size (diameter) and development of root storage organs were recorded.

Induction of polyploidy of in vitro shoots

'Half' and 'whole' shoots were treated with colchicine at 3 different concentrations (0.01 g L^{-1}, 0.1 g L^{-1} and 1.0 g L^{-1}) in colchicine solutions either overnight or for 3 days. After treatment, explants were transferred aseptically onto proliferation medium supplemented with 2 mg L^{-1} BAP. Axillary shoots were allowed to grow out and proliferate over four generations before ploidy levels were verified using flow cytometric analysis.

Verification of ploidy level of colchicine-treated shoots

Leaf samples of maximum 1 cm^2 in size were harvested from axillary shoots and nuclei isolated and stained according to standard methods as described by Partec (Germany) using the

Partec Cystain® kit for nucleus isolation and staining. Ploidy level determinations were carried out using a Partec Cyflow Space ploidy analyser and histograms obtained for each sample. To ensure that plants with leaves verified as tetraploid were solid polyploids, rhizomes were also analysed. Non-chimeric polyploid plants were proliferated using the methods outlined above for further evaluation.

Evaluation of selected horticultural characteristics

Various horticultural characteristics including stomatal distribution, plant height, leaf length and width, growth rate and biomass of leaves and rhizomes, were evaluated. Epidermal peels were produced by applying a layer of clear nail varnish to the adaxial surface of the leaf, allowed to dry and then peeled off using sticky tape which was subsequently stuck onto a microscope slide and viewed. Number of stomata per square mm were counted using a Nikon E400 Eclipse microscope at 400x magnification. Twelve plants were analysed, with five fields of view recorded and averaged for each ploidy level. Biomass was calculated by drying leaves for 3 days in a drying oven (70°C) and comparing with the fresh weight of harvested leaves.

Statistical analysis

The experimental layout for all trials was a complete randomised design (CRD) with 3 replications with a minimum of twelve plants per replicate. Student's t test was carried out to determine significant differences in the results obtained at 5% significance level.

RESULTS AND DISCUSSION

Effect of growth regulators and explant type on in vitro shoot proliferation of *Siphonochilus aethiopicus* shoot cultures

None of the sterilisation methods investigated produced significantly better results than any other in terms of promoting successful initiation of rhizome buds into the aseptic environment. As such, any further investigations used disinfestation procedures which entailed a 30 sec wash in 70% [v/v] ethanol, followed by 30 min in a 1% [v/v] CaOCl (680 g kg^{-1} active ingredient) solution and subsequently 3 rinses with sterile distilled water prior to placing rhizome buds onto the culture medium. Uncontaminated buds were initially transferred to a nutrient medium comprising MS nutrients and vitamins, 30 g L^{-1} sucrose and solidified with 3 g L^{-1} Gelrite. The medium was supplemented with 1 mg L^{-1} BAP to induce shoot proliferation; however, after 4 generations, the multiplication rate remained exceptionally low, 1.54. The best shoot proliferation rate was obtained when shoots were transferred to the same medium supplemented with 2 mg L^{-1} BAP whilst the multiplication rate of shoot cultures remained below 2.0 for all the other growth regulator combinations tested (Table 1). Since shoot cultures showed extremely slow proliferation rates, in attempts to increase the rate, shoots were longitudinally sectioned (termed 'half' shoots) in an attempt to break apical dominance and thereby increase multiplication. The multiplication rate was significantly increased and an average proliferation rate of 4.38 was obtained on medium supplemented with 2 mg L^{-1} BAP. In all instances, medium containing BAP only, resulted in higher multiplication rates than any of the other treatments. A very high percentage of leaf senescence was observed in shoot cultures where KIN was used as growth regulator compared with any of the other treatments.

Effect of plant size and the presence of roots on acclimatisation of tissue cultured plants

Tissue culture methods provide a means for the proliferation of the species but any micropropagation method's success is dependent on whether or not the in vitro shoots can be acclimatized to ambient conditions. In our study, both the size of the plantlets (shoot height and rhizome diameter) and the presence or absence of roots on deflasking were identified as potential limiting factors to successful acclimatization. For both shoots planted out without roots and for plantlets with roots a 87% success survival rate was achieved. For all horticultural characteristics evaluated, there were significant increases in plant height, leaf width and length as well as number of stems per plant and rhizome diameter from plants with roots 3 months after

deflasking (Table 2). Plantlets originating from both shoots without roots and plantlets with roots, showed development of new rhizomes from the original rhizome. From the results obtained, acclimatization success and development of root storage organs do not appear to be affected by the size of the explant or the presence/absence of roots on deflasking. Plantlets originating from rootless shoots were of a similar size to those deflasked with roots 6-8 weeks after transfer to potting bags. A considerable benefit of being able to plant out very small, rootless tissue culture shoots is the major cost- and time-saving obtained if the final step (plant growth and rooting) in the tissue culture protocol can be eliminated. There was no difference between diploid and tetraploid plants in terms of acclimatization success.

Effect of colchicine treatments on polyploidy induction in *Siphonochilus aethiopicus* explants

Polyploidy was induced in 'half' shoots only and furthermore, only the colchicine treatment at a concentration of 0.1 g L^{-1} over a period of 3 days, resulted in the induction of tetraploids and then only at a rate of 4.44% of the shoots treated (Table 2). There was no difference in plant survival rate between treatments and all polyploid shoots survived. No tetraploids were induced for the overnight treatment at the same concentration. No mixoploids or non-chimeric polyploids were induced in shoots treated with 0.01 g L^{-1} colchicine over 3 days and a similar trend was observed for the overnight treatment at the same concentration. Diploid:tetraploid mixoploids were detected for all treatments except the overnight 1 g L^{-1} treatment. No tetraploid:octoploid mixoploids or octoploids were detected for any of the treatments. Mortality rate increased with increasing concentration and exposure time.

Effect of polyploidy on selected horticultural characteristics

A summary of the comparison of diploid and tetraploid lines for selected horticultural traits is presented in Table 4.

1. Stomatal distribution in leaves.

Stomatal distribution in tetraploids was significantly lower than in diploids with the diploids showing on average 73 stomata per square millimetre compared with 40 stomata per square millimetre for the tetraploids, i.e. 1.8X increase in stomatal density. This phenomenon is commonly observed in polyploid plants and lower stomatal density is reported to be indicative of improved adaptation to such environmental stressors such as water stress (Li et al., 1996; Maherali et al., 2009) and changes in atmospheric CO_2 concentration (McElwain and Chaloner, 1995; Kürschner et al., 2008).

2. Biomass of leaves.

Leaves harvested from tetraploid plants had a significantly higher biomass compared with diploid progenitors. It is expected that the rhizomes will also have a higher biomass and, in the case of the rhizomes, this could have significant consequences in terms of not only rhizome yield per plant and therefore per hectare, but also the yield of secondary metabolites. In medicinal plant species, the leaves, stems, flowers, and roots are often the parts used in traditional medicines as they are the source of the active compounds and *S. aethiopicus* is no exception (Zhang et al., 2008). The increased biomass of polyploid plants is therefore a very attractive characteristic in plant improvement and further study on yield and active component composition is ongoing.

3. Plant morphology and growth rate.

Although there were no significant differences in plant height when diploid and tetraploid plants were planted out for acclimatization (plants averaged a height of 30 mm regardless of ploidy level), there were marked differences in leaf morphology. Tetraploid plants had significantly shorter and broader leaves than the diploids. However, 3 months after establishment, tetraploid plants had significantly longer leaves than the diploids, averaging 145 mm in length compared to 67. Leaf width, however, was significantly broader in tetraploids. The tetraploid plants also appeared to have a faster growth rate than the diploid controls. Even though

diploid and tetraploid plants of the same height were planted out, 3 months after planting, tetraploid plants were significantly taller than diploids (211 mm vs 55 mm, average values). Similar results were obtained when polyploidy was induced in culinary ginger, *Zingiber officinale* (Smith et al., 2004; Kun-Hua et al., 2011). Tetraploid plants had significantly larger rhizomes than the diploid plants for the tetraploids compared with the diploids. Interestingly, the tetraploids had, on average, 3.25 stems per plant compared with the diploid plants which had an average of 1.25 stems per plant 3 months after planting. Morphological differences between induced tetraploids and their diploid progenitors of a wide variety of plant species have long been reviewed (Gates, 1909; Stebbins, 1947; Stebbins, 1971; Levin, 2002; Knight & Beaulieu, 2008) and several agriculturally and economically-important tuberous/rhizomatous species, i.e. potato, sweet potato, yam, banana, etc., are polyploid.

CONCLUSIONS

A method for successful in vitro production of tetraploids was developed for *Siphonochilus aethiopicus*. Furthermore, acclimatized tetraploid *S. aethiopicus* plants were morphologically distinct from the diploids in that they had larger leaves and a higher growth rate as measured 3 months after deflasking. Tetraploid plants had fewer stomata than the diploids. Future investigations will include the effect of polyploidy on both rhizome and essential oil yield as well as potential effects on the composition of secondary metabolites.

ACKNOWLEDGEMENTS

The Agricultural Research Council is thanked for financial support. This work is based on the research supported by the National Research Foundation.

Literature Cited

Gates, R. (1909). The stature and chromosomes of Oenothera gigas De Vries. Archiv für Zellforschung 3, 525–552.

Hutchings, A. (1996). Zulu medicinal plants: an inventory. University of Natal Press, Pietermaritzburg, South Africa.

Knight C.A. and Beaulieu, J.M. (2008). Genome size scaling through phenotype space. Ann. Bot. 101, 759–766.

Kun-Hua, W., Jian-Hua, M., He-Ping, H. and Shan-Lin, G. (2011). Generation of autoteraploid plant of ginger (Zingiber officinale Rosc.) and its quality evaluation. Pharmacogn. Mag. 7, 200-206.

Kürschner, W.M., Kvaček, Z., and Dilcher, D. L. (2008). The impact of Miocene atmospheric carbon dioxide fluctuations on climate and the evolution of terrestrial ecosystems. Proc. Natl. Acad. Sci. USA 105, 449–453.

Levin, D.A. (2002). The role of chromosomal change in plant evolution. Oxford University Press, New York, USA.

Li, W.L, Berlyn, G.P. and Ashton, P.M.S. (1996). Polyploids and their structural and physiological characteristics relative to water deficit in Betula papyrifera (Betulaceae). Am. J. Bot. 83, 15–20.

Maherali, H., Walden, A.E. and Husband, B.C. (2009). Genome duplication and the evolution of physiological responses to water stress. New Phytol. 184, 721–731.

McElwain, J.C. and Chaloner, W.G. (1995). Stomatal Density and Index of Fossil Plants Track Atmospheric Carbon Dioxide in the Palaeozoic. Ann. Bot. 76, 389-395.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Smith, M.K., S.D. Hamill, S.D., Gogel, B.J. and Severn-Ellis, A.A. (2004). Ginger (*Zingiber officinale*) autotetraploids with improved processing quality produced by an in vitro colchicine treatment. Aus. J. Exp. Agric. 44, 1065-1072.

Stebbins, G.L. (1947). Types of polyploidy: their classification and significance. Adv.Genet. 1, 403–429.

Stebbins, G.L. (1971). Chromosomal evolution in higher plants. Addison-Wesley, London, UK.

Van, Wyk. B.-E. and Gericke, N. (2000). People's plants: A guide to useful plants of Southern Africa. Briza Publications, Pretoria, South Africa.

Zhang, H.F., Zheng, H., Chen, X.H., and Guo, B.L. (2008). Research progress of polyploid medicinal plants. Hebei J.For. Orch. Res., 3:169–72.

Tables

Table 1. Effect of plant growth regulator on multiplication rate of different explants ('whole'- and 'half' shoots) of in vitro *Siphonochilus aethiopicus* over a period of 24 weeks (3 replicates, n=30 per replicate, P≤0.05). Different superscripts indicate a significant difference at P≤0.05.

Plant growth regulator Composition (mg L^{-1})	Explant type	
	'whole' shoots	'half' shoots
1 BAP	1.74[a]	1.94[b]
2 BAP	3.32[a]	4.41[b]
3 BAP	1.47[a]	1.74[b]
1 BAP + 1 NAA	1.63[a]	1.78[a]
2 BAP + 1 NAA	2.06[a]	2.14[a]
4 BAP + 1 NAA	1.46[a]	1.57[a]
1 KIN	1.40[a]	1.56[ab]
2 KIN	1.52[a]	1.66[a]
3 KIN	1.43[a]	1.56[ab]
1 KIN + 1 NAA	1.35[a]	1.38[a]
2 KIN + 1 NAA	1.91[a]	2.01[a]
4 KIN + 1 NAA	1.39[a]	1.43[a]

Table 2. Comparison of various factors potentially impacting on the survival of in vitro shoots and plantlets *of Siphonochilus aethiopicus* (n = 30, 3 replicates). For each trait, different superscripts indicate a significant difference at P≤0.05 either at planting or after 4 weeks acclimation.

Factor	Plantlets with roots (at planting)	Shoots without roots	Plantlets with roots (4 weeks acclimation)	Shoots without roots
Hardening-off success rate	-	-	87.7[a]	87.4[a]
Average shoot height (mm)	53.2[a]	40.8[ab]	114.2 (115%)[a]	80.4 (97%)[b]
Average root length (mm)	-	-	170.4[a]	90.2[b]
Percentage plants developing root storage organs	-	-	62.5[a]	60.7[a]
Average rhizome diameter increase (mm)	6.4[a]	4.2[b]	9.2 (44%)[a]	5.5 (30%)[b]

Table 3. Effect of colchicine concentration and exposure time on polyploidy induction in in vitro shoots of *Siphonchilus aethiopicus.* Leaf explants of approximately 1cm^2 were harvested from in vitro shoots for analysis.

Imbibition time	Treatment (g L^{-1})	Percentage 2n	Percentage 2n/4n	Percentage 4n	Percentage 4n/8n	Percentage 8n
Overnight (n=200)	0.01 (n=133)	99.25	0.75	0	0	0
	0.1 (n=56)	73.21	26.79	0	0	0
	1.0 (n=11)	100	0	0	0	0
3 days (n=141)	0.01 (n=89)	100	0	0	0	0
	0.1 (n=45)	86.67	8.89	4.44	0	0
	1.0 (n=11)	71.43	28.57	0	0	0

Table 4. Effect of induced polyploidy on selected horticultural characteristics of *Siphonochilus aethiopicus* plantlets (3 replicates, n=12 per replicate). For each trait, different superscripts indicate a significant difference at P≤0.05.

Horticultural trait	Diploid	Tetraploid
Stomatal distribution (mm^2)	73^a	40^b
Plant height at exflasking (mm)	30^a	30^a
Plant height after 3 months (mm)	55^a	211^b
Leaf width at planting (mm)	8^a	16^b
Leaf length at planting (mm)	58^a	45^b
Leaf width after 3 months (mm)	13^a	29^b
Leaf length after 3 months (mm)	68^a	145^b
Number of stems per plant after 3 months	1.25^a	3.25^b
Rhizome diameter (mm)	10^a	18^b

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

CHAPTER 7: CONCLUDING REMARKS AND FUTURE RESEARCH DIRECTIONS

7.1 Introduction

Southern Africa's vast plant diversity of almost 30 000 angiosperm species accounts for 10% of the world's flowering plants. The area is also rich in cultural diversity with a history of plant use and ethnobotanical knowledge. Southern Africa's plants have been used in beverages, as important medicinal preparations as well as in traditional arts and crafts. Even though the traditional knowledge base has been vastly reduced due to changes and moves towards modern health care, education, urbanization and moves towards agricultural production away from subsistence farming, the inherent traditional knowledge systems still remain. Fortunately, a vast amount of this knowledge has been formally recorded (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; van Wyk and Gericke, 2000) by champions of southern African ethnobotany. Although scientific study and documentation remains under-developed, this study area has relatively recently received a vast amount of impetus with several research groups making great strides towards research in indigenous plant knowledge. Van Wyk and Gericke (2000) describe how this impetus has rapidly advanced with the collaborative research efforts of government departments, science councils, tertiary education institutions, communities, traditional healers and farmers as well as business developers. These kinds of interactions have led to various plant-derived natural products being developed and marketed on both local and international markets.

Traditional knowledge is an important aspect of any plant and crop improvement programme as it lays the foundation for development. Southern Africa's diverse plant species with their enormous potential, lend themselves to improvement. New crop advancement is required in order to establish opportunities for the development of sustainable livelihoods, particularly in the rural sector. One such mechanism is through the generation of induced polyploids and this thesis presents case studies of species of different familial origins with polyploid development potential. The results of polyploidisation potentially represent important plant breeding attributes and therefore further promotes the use of polyploids in agriculture. It must be noted, however, that consideration of the socio-economic aspects (such as benefit sharing and indigenous knowledge systems) of new crop uptake should be taken into account especially where there is a history of usage.

7.2 Summary of research findings

Although artificial induction of polyploidy has been carried out since the 1930s, and several plant traits have been improved upon, there is still enormous scope for development of improved plant varieties – no more so than with indigenous southern African species. Doubling the chromosome complement

artificially has previously been reported for numerous species across a wide range of plant families. As highlighted in Chapter 2, although the effects of chromosome doubling are not predictable they may be visible morphologically, biochemically, or not at all. Phenotypic advantages may include morphological changes, physiological changes as well as metabolic changes. Other traits such as resistance to water stress and pathogens, improved vase-life and larger vegetative and reproductive structures are often the target of breeders and could potentially stimulate the use of polyploids in agricultural crop development. The current studies have shown that a variety of characteristics can be affected and furthermore, enhanced, through induced polyploidy using colchicine, a mitotic spindle inhibitor. Apart from species constraints, conditions for successful polyploidy induction need to be established. Duration of exposure as well as the mutagen (colchicine or other mutagen) concentration are key to successful polyploidy induction and these were established for each case study. Verification of polyploidy induction is generally carried out using flow cytometry which negates the three dimensional and artifact effects of chromosome visualisation using microscopy.

For all species used in this study polyploidy was successfully induced and verified using flow cytometry and these are the first reports published for each species. The polyploids of each species were evaluated for morphological and physiological changes pertaining to floriculture (*Crocosmia aurea*), enhanced nutritional value and nematode tolerance (*Plectranthus esculentus*), essential oil content and bioactivity (*Tetradenia riparia*) and rhizome size (*Siphonochilus aethiopicus*). For each case study, several questions have arisen and further research is required to (better) understand changes brought about by induced polyploidisation events. These are summarized and highlighted below:

1. *Crocosmia aurea*

   Induced polyploidy was traditionally studied with the aim of crop domestication in terms of specific fruit/flower size breeding targets. Several polyploid cut flower and ornamental plant species are now standard commercial varieties due to their enhanced size. There are a number of economically-important Iris species which are important cut-flower and/or ornamental crops. These include a number of Freesia, Gladiolus, Watsonia and Iris species, all of which have been improved using chromosome doubling and/or conventional breeding practices. Falling stars iris, *Crocosmia aurea* is a member of the Iridaceae family and has enormous ornamental potential. The current studies showed that there was a marked improvement in flower size, although the number of flowers per inflorescence stem was reduced. Further, although polyploidy can lead to improvements in fertility, the induced polyploids did not set seed. It is speculated that this could possibly be due to altered flower structure and therefore reduced access to pollinators. For induced polyploids of *Crocosmia aurea* highlighted in Chapter 3, unanswered questions regarding vase-life effects and the mechanism/s of potentially enhanced longevity remain to be studied.

2. *Plectranthus esculentus*

Plectranthus esculentus (Family: Lamiaceae), or Livingstone potato, is an edible tuberous vegetable which originated in Africa. Although the tubers are edible, limited crop improvement has been achieved in this under-utilised crop with enormous potential. Further, the crop is highly susceptible to rootknot nematode which globally causes extensive losses to a host of crops. In this study induced tetraploids were found to be highly tolerant to rootknot nematode and were also found to have improved nutritional (starch) value compared with the diploid controls. This is one of very few reports on the development of induced polyploids exhibiting nematode tolerance and has huge potential for other crops and further molecular breeding studies considering the extent of nematode damage to crops and resultant crop and income losses world-wide. The mechanism of tolerance is currently unknown and is proposed to either be biochemical or physical in nature. Investigations are underway to determine if there are any biochemical changes in response to nematode challenge as well as histopathological studies to determine if there are physical deterrents to nematode entry. The identification of simple yet linked markers for tolerance would facilitate screening, selection and breeding of tolerant cultivars – not only for this species but also for other crops highly susceptible to rootknot nematode.

3. *Tetradenia riparia*

Ginger Bush, *Tetradenia riparia* (Family: Lamiaceae), is an aromatic shrub that occurs throughout tropical Africa and in South Africa, has traditionally been used in the treatment of cough, dropsy, diarrhoea, fever, headaches, malaria, and toothache. The essential oils are also used in the perfume industry. In this investigation, induced tetraploids contained at least twice the amount of essential oil compared with the diploids and the biochemical profile was significantly altered in the tetraploids compared with the diploids. Results indicated that the essential oils from tetraploid plants significantly inhibited the *in vitro* growth of *Geotrichum candidum*, an important fungus of post-harvest concern, at different spore concentrations and incubation times. Therefore, tetraploid plants produce essential oil with potential to mitigate post-harvest diseases. Further research needs to be carried out to determine the potential to develop environmentally-friendly products arising from *T. riparia*. This is the first report on the bioactivity of *T. riparia* essential oil against any fungus.

4. *Siphonochilus aethiopicus*

   Siphonochilus aethiopicus (wild ginger family Zingiberaceae) is one of only several thousand plant species used in traditional medicinal preparations in South Africa, albeit one of the most widely-used and therefore scarce, species. The plant is threatened with extinction and is already extinct in the wild in the KwaZulu-Natal Province and is increasingly threatened in the Mpumalanga Province. The cone-shaped rhizomes and fleshy roots are extremely popular and are widely used in traditional medicines which include treatments for asthma, hysteria, colds, coughs and flu, as well as malaria, amongst others. Previous reports on induced polyploids in culinary ginger, *Zingiber officinale*, showed that rhizome size and yields could be substantially improved, hence the study on *S. aethiopicus*. Preliminary findings showed that polyploid plants had a higher growth rate and rhizome size compared with their diploid progenitors – even at a relatively young age. Polyploids also showed a tendency to be more cold-tolerant during the winter months and did not become completely deciduous, however this needs further investigation. Such changes in phenology could indicate higher assimilation rates during the colder winter months and therefore greater propensity for storage of assimilates and therefore faster growth rate of rhizomes. An assessment of changes in bioactive compounds will need to be carried out to determine the effect of polyploidy on these compounds.

7.3 Strategies for future research in terms of breeding and molecular breeding strategies for the elucidation of control mechanisms and gene expression

Polyploid plants have more than two complete sets of chromosomes and are common in the angiosperms (Stebbins, 1947; Gregory and Mable, 2005). Evolution and speciation have arisen as a result of naturally-induced polyploidisation events and plant breeders have sought to harness this natural phenomenon for the improvement of useful plant species Soltis *et al*. (2009). Many crops including cereals, legumes, industrial plants, tuberous plants, fruit trees and forage grasses are polyploid, both natural and induced. Novel phenotypes and a higher degree of variation compared with the parental diploids is often, but not always, observed following polyploidisation events (Otto and Whitton, 2000). Phenotypic differences may include alterations in morphology, physiology and biochemistry and with these alterations come traits such as drought tolerance, pest and disease tolerance, alterations in flowering time as well as larger vegetative (e.g. tubers) and reproductive (e.g. flowers and fruits) organs, some of these conferring increased ability to withstand non-optimal conditions. This has led to the development of several polyploids of agricultural importance (Renny-Byfield and Wendel, 2014).

In the current investigation, based on the incredible diversity of differences across all the case studies summarised above, further research will require deciphering of biological processes and their control mechanisms on a case-by-case basis. New genomics tools through the use of genome-wide and targeted

approaches should assist in unravelling such biological mechanisms for use in breeding and improvement programmes. Knowledge accumulated on polyploid development and subsequent performance at genome level can assist plant breeders in designing strategies for crop improvement using induced polyploidy. Madlung (2013) described three major advantages of polyploids over diploids. The first being that the increased allele number should mask deleterious effects of recessive mutations thereby ensuring against loss of viability. Secondly, heterosis allows the polyploid's subsequent generations to have transgressive performance compared with the diploid where hybrid vigour can be drastically reduced due to homologous recombination. A third advantage, and the most important in terms of polyploid crop performance is the potential that duplicated gene copies evolve and assume altered or even new functions which would allow for expansion into niche environments or increased ability to respond to biotic and/or abiotic stress events.

Advances in the field of genomics as well as genome sequencing are now beginning to provide the opportunity for discovering and monitoring the molecular basis of polyploidisation. It is thought to be correlated with genomic sequence and changes in gene expression and as such there are vast differences between diploids and polyploids in terms of gene expression (Adams and Wendel, 2005; Jackson and Chen, 2010; Birchler, 2012; Hegarty *et al.*, 2013). There is a slew of recent literature highlighting the fact that advantageous or deleterious changes in polyploids compared with their progenitors are due to changes in transcriptomes, genomic architecture, gene silencing or activation as well as loss of DNA making polyploids markedly different from their diploid progenitors (Soltis *et al*., 2009; Olsen and Wendel, 2013; Renny-Byfield and Wendel, 2014; Zhang *et al*., 2014; Borrill *et al*., 2015). Several methods including *in situ* hybridization, marker-based genetic mapping, comparative genome analysis, high-throughput sequencing and high resolution melting analysis, and others, have all been used for genome analysis of polyploids. Northern hybridization and CDNA-AFLPs (amplified fragment length polymorphism transcript profiling), SSCP (single strand conformational polymorphism), microarrays and high throughput RNA sequencing are used in the quest for exploring and understanding gene expression and regulation analysis (Aversano *et al.*, 2012). The discovery of SNPs (single nucleotide polymorphisms) has led to the development of high throughput genotyping of large populations where genetically-mapped SNPs are now commonly used in breeding programmes (Bundock *et al*., 2009). SNP discovery is also exceptionally useful for sequencing individuals with contrasting phenotypes (such as in the case of polyploids which are phenotypically distinct from their diploid progenitors) to identify specific markers for a trait/s of interest thereby facilitating breeding towards specific outcomes.

With technology improving at a rapid rate and the application of genomic tools, polyploidy research is expanding rapidly such that studies involving multidisciplinary approaches will facilitate the translation of genomic knowledge gained into practical application. Next-generation sequencing in particular will not only advance gene and marker discovery but will also facilitate the quantification of gene expression

– such as those coding for resistance, secondary metabolites and assimilation. New genomic resources will increase the ability to identify, understand and even facilitate the manipulation of allele variation and hence phenotypic variation thus allowing this variation to be made available for breeding, selection and improvement of crops – although this is as yet not fully understood (Moghe and Shiu, 2014).

Although there remain a number of unanswered questions regarding polyploidy and the success of polyploid plants compared with diploids, particularly the genetic basis of what constitutes success, improvement of crops, including South Africa's plant resources, using artificial polyploidy induction has the potential for developing improved varieties. These polyploid varieties may be resistant to abiotic and biotic stressors associated with climate change (drought, pests, diseases etc.) and at the same time address the issue of food security in a world where food resources are becoming scarcer.

REFERENCES

Adams, K. and Wendel, J. (2005) Novel patterns of gene expression in polyploid plants. Trends in Genetics, 21: 539-543.

Aversano, R., Ercolano, M.R., Caruso, I., Fasano. C., Rosellini, D. and Carputo, D. (2012). Molecular Tools for Exploring Polyploid Genomes in Plants. International journal of Molecular Science, 13, 10316-10335.

Birchler, J. (2012) Insights from paleogenomic and populations studies into the consequences of dosage sensitive gene expression in plants. Current Opinion in Plant Biology, 15: 544-548.

Borrill, P., Adamski, N. and Uauy, C. (2015) Genomics as the key to unlocking the polyploid potential of wheat. New Phytologist, doi: 10.1111/nph.13533.

Bundock, P.C.; Eliott, F.G.; Ablett, G.; Benson, A.D.; Casu, R.E.; Aitken, K.S.; Henry, R.J. Targeted single nucleotide polymorphism (SNP) discovery in a highly polyploid plant species using 454 sequencing. Plant Biotechnol. J. 2009, 7, 347–354.

Gregory, T.R., and Mable, B.K. (2005). Polyploidy in plants. In The Evolution of the Genome, T.R. Gregory, ed. (San Diego: Elsevier), pp. 427–517.

Hegarty, M., Coate, J., Sherman-Broyles, S., Abbot, R. and Doyle, J. (2013) Lessons from natural and artificial polyploids in higher plants. Cytogenetic and genome research, 140: 204-225.

Hutchings, A. (1996). Zulu Medicinal Plants. Natal University Press, Pietermaritzburg.

Jackson, S. and Chen, Z. (2010) Genomic and expression plasticity of polyploidy. Current Opinion in Plant Biology, 13: 153-159.

Madlung, A. (2013) Polyploidy and its effect on evolutionary success: old questions revisited with new tools. Heredity, 110(20): 99-104.

Moghe, G. and Shiu, S-H. (2014) Annals of the New York Academy of Sciences, 1320: 16-34.

Olsen, K. and Wendel, J. (2013) A bountiful harvest: Genomic insights into crop domestication phenotypes. Annual Review of Plant Biology, 64: 47-70.

Otto, S. and Whitton, J. (2000) Polyploid incidence and evolution. Annual Review of Genetics, 34: 401-437.

Renny-Byfield, S. and Wendel, J. (2014) Doubling down on genomes: Polyploidy and crop plants. Special invited paper. American Journal of Botany, 101: 1711-1725.

Soltis, D., Albert, V., Leebens-Mack, J., Bell, C., Paterson, A., Zheng, C., Sankoff, D., dePamphilis, C., Wall, P. and Soltis, P. (2009) Polyploidy and angiosperm diversification. American Journal of Botany, 96: 336-348.

Stebbins, G. (1947) Types of polyploids: their classification and significance. Advances in Genetics, 1: 403-429.

Van Wyk, B-E. and Gericke, N. (2000) People's Plants: a guide to useful plants of southern Africa. (351 pages). Briza Publications, Pretoria.

Watt, J. and Breyer-Brandwijk, M. (1962) The Medicinal and Poisonous Plants of Southern and Eastern Africa. E & S Livingstone Publishers, London.

Zhang, X., Deng, M. and Fan, G. (2014) Differential transcriptome analysis between *Paulownia fortunei* and its synthesized autopolyploid. International Journal of Molecular Science, 15: 5079-5093.

APPENDIX

APPENDIX

South African Journal of Botany 88 (2013) 367–372

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea*

K. Hannweg [a,b,*], A. Sippel [a], I. Bertling [b]

[a] *Agricultural Research Council, Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, South Africa*
[b] *Horticultural Science, School of Agricultural, Earth and Environmental Sciences, Private Bag X01, University of KwaZulu-Natal, Scotsville, 3209, South Africa*

ARTICLE INFO

Article history:
Received 11 June 2013
Received in revised form 5 September 2013
Accepted 6 September 2013
Available online 30 September 2013

Edited by M.E. Light

Keywords:
Chromosome doubling
In vitro breeding
Iridaceae
Micropropagation

ABSTRACT

South Africa is home to approximately 10% of the world's flora, many of which are endemic to the country. A large number of South African genera have been improved for horticultural use and many of these are economically important as cut flowers or ornamentals on international markets. The genus *Crocosmia*, an attractive member of the family Iridaceae, has potential both as an ornamental plant and for cut flower production, although market potential of the species may be increased by improving the size of the flowers and inflorescence. Polyploidy has been used as a tool in the improvement of ornamental plants and has led to the development of several improved ornamental species. This study established a micropropagation protocol for *Crocosmia aurea*, using seed as the source material. Tetraploidy was induced by treating seeds with colchicine. These seeds were subsequently germinated and multiplied *in vitro* using the established protocol. The resulting tetraploid plantlets were successfully hardened-off and used to study the effect of the induced tetraploidy on the plant characteristics. The tetraploid (4n) plants were found to have longer, wider leaves as well as longer inflorescence stems and fewer, but larger, flowers than their diploid (2n) counterparts. These polyploid selections have potential in the ornamental/floriculture trade.

1. Introduction

The family Iridaceae comprises 65 genera and over 2000 species, of which 38 genera and more than half the species occur in South Africa. The plants are cormous or rhizomatous and have sword-like leaves. In general, the flowers are attractive and many species are important garden ornamentals across the globe. Although numerous species of the family have been improved by plant breeders throughout the world (Niederwieser et al., 2002; Ascough et al., 2009), there are many more species with potential for further horticultural development. *Crocosmia aurea* is no exception. This iris has bright orange-red flowers which appear from January to June on a branched inflorescence, following which a fleshy seed capsule containing purple-black seeds develops. The plant is wide-spread in the eastern parts of South Africa, where it occurs predominantly in moist areas from the coast to 2000 m above sea level (Pooley, 1993). Owing to its inherent beauty, *C. aurea* was selected for investigation of its potential as an ornamental plant and for cut flower production.

Conventional breeding has resulted in substantial improvement of genera within the family Iridaceae and many of these, such as *Gladiolus*, *Iris* and *Freesia* are important cut flowers on both local and international markets. The development of polyploid (chromosome doubling) induction protocols offers enormous potential for further improvement in the family. Naturally-occurring polyploidy is a phenomenon that has provided an important pathway for evolution and speciation in plants. Although the first polyploid was discovered over a century ago, the genetic and evolutionary implications of polyploidy are still being elucidated (Yang et al., 2011). The relative ease with which artificial induction of polyploidy can be achieved provides an opportunity for using this naturally-occurring phenomenon as a valuable tool in plant breeding programmes, where polyploidy has been used extensively as a tool for creating novelty in ornamental crops (Levin, 1983; Väinölä, 2000; Ascough et al., 2008). In general, tetraploids have larger flowers and fruit than their diploid counterparts and furthermore, because of their altered blooming periods, may have wider harvesting and marketing windows (Levin, 1983). These factors are particularly important in ornamental plants and cut flowers, when the potential for commercialisation is addressed. The artificial induction of polyploidy has been reported for a number of South African iridaceous genera, including *Watsonia* (Ascough et al., 2007, 2008) and *Gladiolus* (Suzuki et al., 2005).

Because naturally-occurring polyploid genotypes are usually unavailable, polyploidy is typically induced in breeding programmes

* Corresponding author at: Agricultural Research Council, Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, South Africa. Tel.: +27 13 753 7000; fax: +27 13 752 3854.
E-mail address: Karin@arc.agric.za (K. Hannweg).

0254-6299/$ – see front matter © 2013 SAAB. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.sajb.2013.09.005

through mitotic spindle inhibition or microtubule polymerization, often by exposure to colchicine (Caperta et al., 2006). Polyploid induction depends on the concentration of colchicine, the duration of exposure, explant type, and tissue penetrability (Allum et al., 2007). Colchicine-induced polyploidy is characterized by low induction rates and a high frequency of chimaeras or mixoploids which must be screened out of the population; this is most commonly achieved through flow cytometry analysis (Galbraith et al., 1997). If colchicine-induced pure tetraploids are not produced, rapid *in vitro* proliferation can be used to segregate pure tetraploids from chimaeras, due to the nature of the *in vitro* proliferation system.

Micropropagation has increasingly become a valuable tool for breeders, assisting in releasing new selections and cultivars into the market more rapidly. Ascough et al. (2009) reported that the first published record of *Crocosmia* micropropagation was by Koh et al., 2007. Ovaries and florets of *Crocosmia crocosmiiflora* were cultured with 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP) or kinetin. Callus formed on ovaries at low frequencies (1–28%) with BA and 2,4-D, but rooting was prolific with either NAA or 2,4-D. When florets were used as starting material, roots and corms were induced using a combination of kinetin with either 2,4-D or NAA. These corms produced shoots when kinetin was applied, callus when 2,4-D was used in combination with BA, and corms when kinetin was used in combination with 2,4-D.

The primary objective of this study was to develop a rapid and efficient protocol for the micropropagation of *C. aurea*, to establish methods for polyploid induction and to assess selected floral characteristics of the resulting polyploid plants, with a view to the development and improvement of the species.

2. Materials and methods

2.1. Development of micropropagation protocols

Diploid seeds (obtained from Silverhill Seeds, Cape Town) of *C. aurea* were used as starting material to develop a micropropagation protocol. Five hundred seeds were sterilised for 20 min using a 1% [w/v] calcium hypochlorite solution. The sterilised seeds were rinsed three times with sterile distilled water before being cultured on standard MS medium (Murashige and Skoog, 1962 [MS]) containing 30 g l^{-1} sucrose, adjusted to pH 5.7 and sterilized by autoclaving for 20 min at 121 °C at 1 bar. Each seed was germinated in a glass tube containing 10 ml medium.

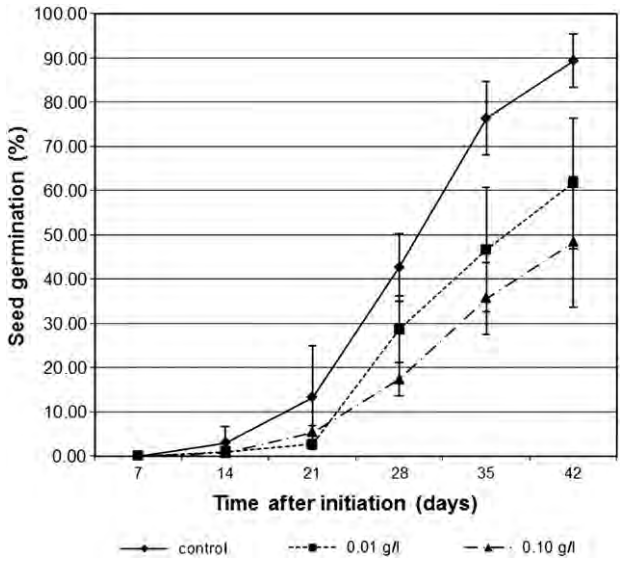


Fig. 1. Effect of colchicine concentration on seed germination of *Crocosmia aurea*.

The germinated seedlings were then transferred to MS medium containing one of four concentrations of BAP – 0.0 μM, 4.4 μM, 8.8 μM or 13.2 μM – to determine the most appropriate medium for plantlet multiplication. Each of the four BAP treatments comprised three replicates with 20 plantlets per replicate. Plantlets were maintained at 25 to 27 °C under a 16/8 h light/dark regime with cool white fluorescent light (81 μmol m^{-2} s^{-1}, Phillips 65 W) and were subcultured every 4 to 6 weeks. Once multiplied and rooted, the medium was rinsed from the roots and plantlets were hardened-off for 4 weeks in a mist bed (housed within a polycarbonate tunnel maintained at 15 to 27 °C) in Speedling® trays containing a 1:1 (v/v) mixture of composted pine bark and coarse river sand, before being planted out into 2 l potting bags containing a 1:1 (v/v) mixture of composted pine bark and sand. Plants were thereafter maintained in a shade house (40% shade cloth) at ambient conditions. Plants were watered daily on an irrigation system and Osmocote® was applied as a slow-release fertiliser.

2.2. In vitro induction of polyploidy

Diploid seeds of *C. aurea* were used as starting material to produce tetraploid plants, with the protocol described above being used to multiply plantlets generated from the treated seed. Five hundred diploid seeds were physically scarified to facilitate colchicine uptake and then treated, under aseptic conditions, with a 25 μM sterile colchicine solution for 3 days or, alternatively, a 0.25 μM solution overnight (based upon results previously obtained in our laboratory). The treated seeds were cultured on the previously determined optimum *in vitro* medium (MS medium supplemented with 4.4 μM BAP). Germination rate was recorded over 6 weeks, as was the final germination percentage. Proliferation rate (number of shoots per 4–6 week subculture interval), shoot height and rooting percentage were measured to determine differences between the two colchicine treatments as well as between diploid and tetraploid shoot cultures. Plants were maintained *in vitro* until they were large enough for ploidy analysis (approximately 12 weeks after treatment) and subculture. The experiment was repeated three times.

2.3. Ploidy analysis and proliferation of confirmed tetraploid plants

The ploidy level of treated seedlings was verified using a Partec PA ploidy analyser (Partec, Germany). Samples were prepared for flow cytometry analysis using approximately 1 cm^2 of leaf tissue. The tissue was macerated with a razor blade in 125 μl of nucleus extraction solution (Partec, Germany), after which the homogenate was filtered through a 50 μm mesh filter. The isolated nuclei were stained with 1250 μl 4′-6-diamidino-2-phenylindole (DAPI) stain (Partec, Germany) prior to commencing flow cytometry analysis. Nuclei isolated from untreated, diploid plantlets were used as a standard. Histograms were analysed using the Partec software package. Seedlings confirmed as tetraploid were proliferated on MS medium containing 4.4 μM BAP under the same conditions described above. Plantlets were hardened-off as described above and used to determine polyploidy effects on various horticultural characteristics.

2.4. Morphological characterisation of tetraploids

In order to evaluate whether polyploidy induction had an effect on the horticultural characteristics of *Crocosmia*, diploid and tetraploid plantlets were proliferated and maintained as described above. The following characteristics were evaluated once the micropropagated plants had hardened-off and reached maturity: leaf width and length, flower diameter, petal width and length, stigma, stamen and anther length, inflorescence diameter and length, as well as flower bud number per inflorescence.

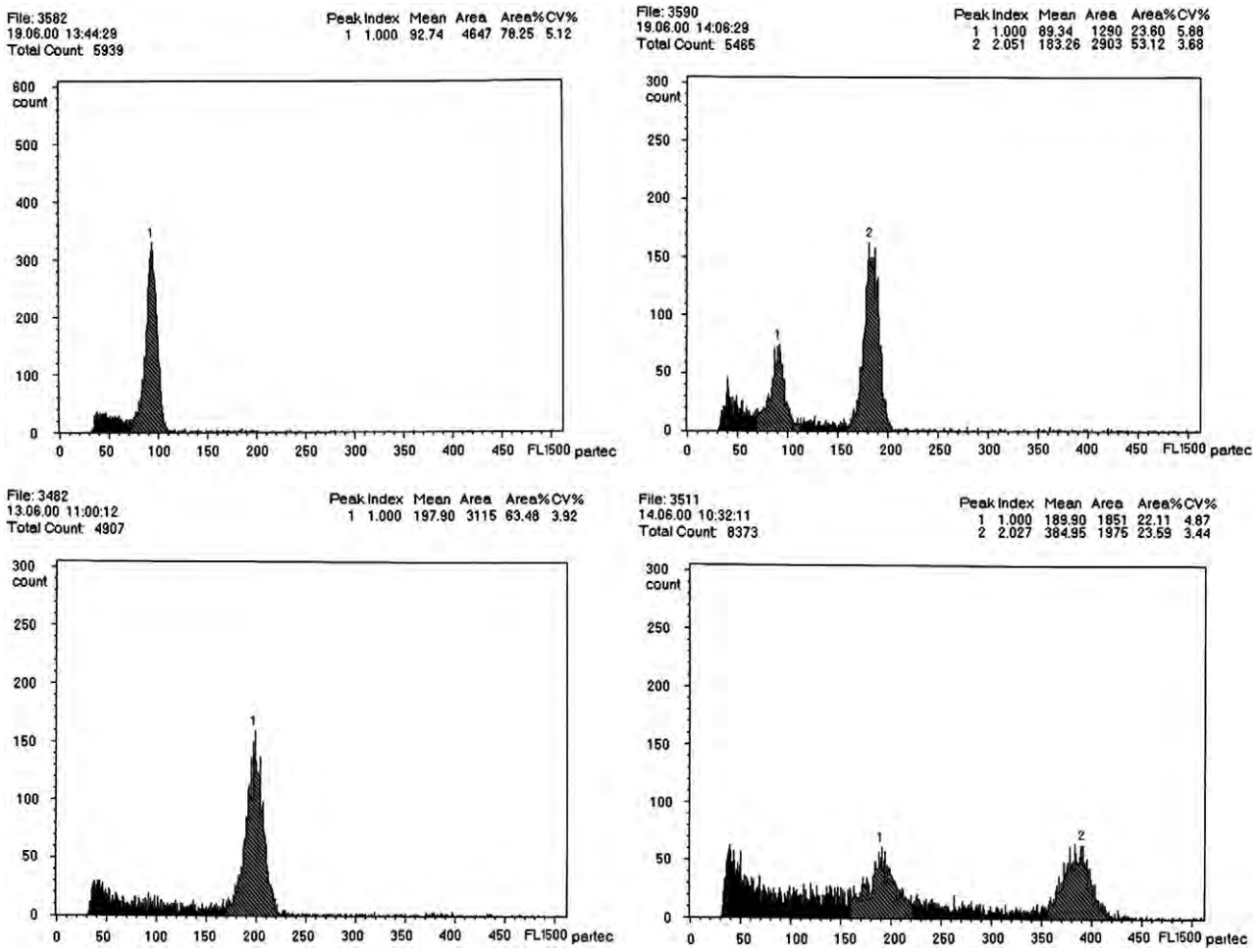


Fig. 2. Representative flow cytometric histograms documenting the ploidy level of nuclei isolated from leaves. A. diploid, B. tetraploid, C. diploid:tetraploid mixoploid, D. tetraploid:octoploid mixoploid.

2.5. Statistical analysis

The experimental layout for all experiments was a complete randomised design (CRD) with 3 replications. An analysis of variance (*t*-test) was performed at the 5% significance level.

3. Results and discussion

3.1. Development of a simple method for in vitro micropropagation of C. aurea

Despite the wealth of available germplasm amongst the Iridaceae, only 40 species from 12 genera have been micropropagated (Ascough et al., 2009). George (1993) and Ascough et al. (2009) presented extensive summaries of bulbous and cormous species which were micropropagated *in vitro*, and subsequently successfully hardened-off and established *ex vitro*. According to Ascough et al. (2007), root and leaf explants of *Watsonia* spp. were incapable of shoot regeneration, but hypocotyl segments were highly regenerative when both an auxin (NAA) and cytokinin (BAP) were present in the medium. However, shoot multiplication was greatest when only BAP (2.2 μM) was added to the medium. Similar results were obtained for *Dierama latifolium* (Page and van Staden, 1985) and *Schizostylis coccinea* (Hussey, 1976), both iridaceous species. In the current study, aseptic cultures of *C. aurea* were readily established using the methods described above. Although BAP at a concentration of 4.4 μM had a tendency to produce a slightly higher multiplication rate (3.35 shoots every 4-6 weeks) than BAP at a concentration of 8.8 μM (3.2 shoots every 4–6 weeks), the difference in treatments was not significant after a period of 4 months. The addition of BAP at a concentration of 13.2 μM gave a shoot multiplication rate of 2.75. Although growth regulator addition usually speeds up the rooting process and increases rooting percentage, as has been reported for several other genera (George, 1993), transfer of *C. aurea* shoots to a growth regulator-free MS medium resulted in root formation on 100% of shoots cultured in this experiment.

Table 1
Effect of *in vitro* colchicine treatments on polyploidy induction in *Crocosmia aurea*.

Treatment	Exposure time	Ploidy (%)				
		Diploids	2n:4n Mixoploids	Tetraploids	4n:8n Mixoploids	Octoploids
Control		100.0	0	0	0	0
0.25 μM	Overnight	53.51	8.77	29.82	3.51	4.39
25 μM	3 days	74.16	9.80	16.04	0	0

Table 2
Comparison after 4 months of the *in vitro* performance of diploid and tetraploid *Crocosmia aurea* plantlets on MS nutrient medium supplemented with 30 g/l sucrose and various concentrations of 6-benzylaminopurine (BAP).

Treatment	0 μM BAP		4.4 μM BAP		8.8 μM BAP		13.2 μM BAP	
	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid
Proliferation rate[a]	2.13[a]	2.25[a]	3.35[b]	3.25[b]	3.20[b]	3.32[b]	2.75[a]	2.63[a]
Shoot height (mm)	45.2[a]	42.3[a]	46.5[a]	45.3[a]	42.5[a]	41.8[a]	43.5[a]	42.1[a]
Rooting (%)	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]	100.0[a]

Different letters denote a significant difference at $p < 0.05$.
[a] Number of shoots per 4–6 week subculture interval.

3.2. Germination rate of colchicine-treated seeds

The germination rate and percentage of seeds treated with 0.25 μM colchicine tended to be higher than that of the 25 μM treatment ($p < 0.05$), although the difference was not statistically significant (Fig 1). However, colchicine had a significant negative effect on germination for both the 0.25 μM overnight and 25 μM three day treatments, when compared with the control. The differences in germination rate manifested five to 6 weeks after initial exposure to colchicine. Similar findings have been reported on a wide range of species (Ramsey and Schmeske, 1998).

3.3. Effect of colchicine on polyploidy induction and in vitro development of induced plantlets

Samples of leaf material were harvested for flow cytometry analysis from *in vitro*-grown shoots 12 weeks after colchicine treatment. Flow cytometry analysis was carried out to determine the ploidy levels of *in vitro* regenerants, which included mixoploids (Fig. 2). Of the plants treated overnight with 0.25 μM colchicine, 29.82% were identified as tetraploids, with 8.77% identified as 2n:4n mixoploids and 3.51% as 4n:8n mixoploids (Table 1). A small percentage (4.39%) was identified as octoploid for the same treatment. For the 25 μM three-day treatment, 16.04 % of the seedlings were identified as tetraploid, with 9.8% being 2n:4n mixoploids and no octoploids identified (Table 1). All mixoploids were discarded to prevent proliferation of an unstable population of such plants. The effectiveness of colchicine application and polyploidy induction *in vitro* not only depends highly on the plant species but also on the colchicine concentration applied, duration of treatment, type of explant, and the penetration of the compound (Allum et al., 2007). Colchicine has been used effectively at both lower (i.e. 0.25 μM for *Lychnis senno* (Chen et al., 2006)) and very high concentrations (i.e. 38,000 μM for *Chaenomeles japonica* (Stanys et al., 2006)). Within the Iridaceae family, polyploidy has been successfully induced in *Gladiolus* spp. (Suzuki et al., 2005) and *Watsonia lepida* (Ascough et al., 2008).

There was no significant difference in *in vitro* shoot height, multiplication rate or rooting between *in vitro* diploid and tetraploid shoots (Table 2). Rooted plantlets, both diploid and tetraploid, were readily hardened-off in the mist bed. There was no difference in survival rate or in plant growth and development between diploid and tetraploid plants. Survival rate was in the order of 98% for both diploid as well as for tetraploid plants.

Table 3
Effect of polyploidy on selected morphological characteristics of *Crocosmia aurea*.

Morphological characteristic	Diploid	Tetraploid
Flower diameter (mm)	62.78[a]	78.2[b]
Width of petal (mm)	8.936[a]	15.04[b]
Length of petal (mm)	31.05[a]	36.72[b]
Length of stamens (mm)	26.07[a]	32.27[b]
Length of stigma (mm)	29.70[a]	29.03[a]
Length of anther (mm)	9.53[a]	8.585[a]
Diameter of inflorescence stem (mm)	3.09[a]	3.54[b]
Length of inflorescence stem (mm)	794.67[b]	577.70[a]
Number of flowers	29.00[b]	21.67[a]

Different letters within a row denote significant difference at $p < 0.05$, $n = 12$.

3.4. Morphological characteristics of tetraploid C. aurea plants

Overall flower size i.e. flower diameter, petal width, petal length, stamen length, diameter and length of the inflorescence stem and the number of flowers on the inflorescence were significantly different for the induced tetraploid plants compared with the diploid plants (Table 3). Overall flower size i.e. flower diameter and petal length and breadth (Fig. 3) and the diameter of the inflorescence stem were significantly larger and thicker, respectively, for the induced tetraploids compared with the diploids. Conversely, the length of the inflorescence stem and number of flower buds per inflorescence was significantly lower for the induced tetraploids. Polyploidy can result in significant cell enlargement, which is particularly desirable for flowering ornamental species. Several reports since the advent of induced polyploidy research on ornamentals describe increases in flower size (Emsweller and Ruttle, 1941; Tulay and Unal, 2010), alterations in inflorescence stem length (Griesbach and Bhat, 1990; Takamura and Miyajima, 1996), and number of flowers per stem (Kafawin and Chen, 1991; Tulay and Unal, 2010) for a wide range of ornamental species, and with differing results. There is no means to predict direction of morphological or physiological change for induced polyploids for a particular species. For *C. aurea*, there appeared to be no significant difference between induced tetraploids and diploids regarding certain reproductive parts of the flower (anther and stigma length), while others (stamens) were significantly longer in the tetraploids than in the diploids. The induced tetraploids appeared to be sterile since no seed was set over the evaluation period compared with the diploids, 100% of which set seed. This could be a physical constraint during pollination due to the altered morphology of the plant parts, since pollen viability studies using acetocarmine staining showed no difference in viability between diploid and tetraploid pollen (results not shown). Changes in flower shape, due to polyploidy, have been reported to attract different species of insects to the polyploid flowers which could preclude pollination (Thompson and Merg, 2008). Although tetraploid plants did not set seed, the *in vitro* micropropagation method developed would facilitate the establishment of a mother-block of polyploid plants (and thereby establish a genebank) which are easily hardened-off. Producers could then successfully vegetatively propagate *C. aurea* through division of corms.

C. aurea is thus readily propagated *in vitro*, and *in vitro* tetraploid induction provides a method for the development of *C. aurea* plants with significantly larger flowers than their diploid counterparts. Preliminary observations of flowering of the tetraploid plants indicated that flowering appears to be initiated several weeks after the diploids. In fact, flowering mimics the vegetative phenological cycle, with the dormant tetraploid corms producing vegetative growth several weeks after the diploids (preliminary observation; data not shown). It has

Fig. 3. Comparison of diploid (A and B) and induced tetraploid (C and D) *C. aurea* flowers. Scale bar represents 1 cm.

previously been reported that tetraploids, which may have a slower growth rate, may flower later or over a longer period than their diploid progenitors (Datta, 1963; Roy and Dutt, 1972; Levin, 1983). This is an attribute of particular interest in ornamental breeding (Weiss, 2002). Furthermore, Kehr (1996) reported that flowers are longer lasting in polyploid plants — vase life has yet to be determined for the tetraploid plants produced in this study. In conclusion, additional research is needed to establish possible alterations in vase life of tetraploids compared with the diploids; phenological studies to determine flowering times are also required to determine a potential increase or decrease of marketing windows.

Acknowledgements

The Agricultural Research Council and the Department of Science and Technology are thanked for funding the investigations. The assistance of Mr Mark Penter with proofreading is acknowledged. Mr Gerrit Visser is thanked for conducting the flow cytometry.

References

Allum, J.F., Bringloe, D.H., Roberts, A.V., 2007. Chromosome doubling in *Rosa rugosa* Thunb. hybrid by exposure of *in vitro* nodes to oryzalin: the effects of node length, oryzalin concentration and exposure time. Plant Cell Reports 26, 1977–1984.

Ascough, G.D., Erwin, J.E., van Staden, J., 2007. *In vitro* propagation of four *Watsonia* species. Plant Cell, Tissue and Organ Culture 88, 135–145.

Ascough, G.D., Erwin, J.E., van Staden, J., 2008. Effectiveness of colchicine and oryzalin at inducing polyploidy in *Watsonia lepida* N.E. Brown. Plant Cell, Tissue and Organ Culture 43, 2248–2251.

Ascough, G.D., Erwin, J.E., van Staden, J., 2009. Micropropagation of iridaceae — a review. Plant Cell, Tissue and Organ Culture 97, 1–19.

Caperta, A.D., Delgado, M., Ressurreição, F., Meister, A., Jones, R.N., Viegas, W., Houben, A., 2006. Colchicine-induced polyploidization depends on tubulin polymerization in c-metaphase cells. Protoplasma 227, 147–153.

Chen, L.P., Wang, Y.J., Zhao, M., 2006. *In vitro* induction and characterization of tetraploid *Lychnis senno* Siebold et Zucc. HortScience 41, 759–761.

Datta, R.M., 1963. Investigations on the autotetraploids of the cultivated and the wild types of jute (*Corchorus olitorius* Linn. and *C. capsularis* Linn.). Zuchter 33, 17–33.

Emsweller, S., Ruttle, M., 1941. Induced polyploidy in floriculture. American Naturalist 75, 310–328.

Galbraith, D.W., Lambert, G.M., Macas, J., Doležel, J., 1997. Current Protocols in Cytometry, Analysis of Nuclear DNA Content and Ploidy in Higher Plants. In: Robinson, J.P. (Ed.), John Wiley and Sons, New York, pp. 7.6.1–7.6.22.

George, E., 1993. Plant Propagation by Tissue Culture, second ed. Exegetics Limited, Edington, England.

Griesbach, R., Bhat, R., 1990. Colchicine-induced polyploidy in *Eustoma grandiflorum*. HortScience 25, 1284–1286.

Hussey, G., 1976. *In vitro* release of axillary shoots from apical dominance in monocotyledonous plantlets. Annals of Botany 25, 1323–1325.

Kafawin, O., Chen, C., 1991. Induction of tetraploid *Lilium longflorium* Thumb plants by colchicine treatment of cultured bulbscale discs. Proceedings of the South Dakota Academy of Science 70, 31–37.

Kehr, A.E., 1996. Woody plant polyploidy. American Nurseryman 183, 38–47.

Koh, J.C., Lee, E.J., Park, H.G., 2007. Effects of several plant growth regulators on callus and corm formation of *Crocosmia crocosmiiflora in vitro*. Flower Research Journal 15, 238–244.
Levin, D., 1983. Polyploidy and novelty in flowering plants. American Naturalist 122, 1–25.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15, 473–497.
Niederwieser, J.G., Kleynhans, R., Hancke, F.L., 2002. Development of a new flower bulb crop in South Africa. Acta Hortiulturae 570, 67–71.
Page, Y.M., van Staden, J., 1985. *In vitro* propagation of *Dierama latifolium*. HortScience 20, 1049–1050.
Pooley, E., 1993. The Complete Field Guide to the Trees of Natal. KZN Flora & Fauna Publications Trust, KwaZulu-Natal, Zululand and Transkei.
Ramsey, J., Schmeske, D., 1998. Pathways, mechanisms and rates of polyploidy formation in flowering plants. Annual Review of Ecology and Systematics 29, 467–501.
Roy, R.P., Dutt, B., 1972. Cytomorphological studies in induced polyploids of *Luffa acutangula* Roxb. Nucleus 15, 181–189.
Stanys, V., Weckman, A., Staniene, G., Duchovskis, P., 2006. *In vitro* induction of polyploidy in Japanese quince (*Chaenomeles japonica*). Plant Cell, Tissue and Organ Culture 84, 263–268.
Suzuki, K., Takatsu, Y., Gonai, T., Kasumi, M., 2005. Plant regeneration and chromosome doubling of wild *Gladiolus* species. Acta Horticulturae 673, 175–181.
Takamura, T., Miyajima, I., 1996. Colchicine-induced tetraploids in yellow-flowered cyclamens and their characteristics. Scientia Horticulturae 65, 305–312.
Thompson, J.N., Merg, K.F., 2008. Evolution of polyploidy and the diversification of plant–pollinator interactions. Ecology 89, 2197–2206.
Tulay, E., Unal, M., 2010. Production of colchicine-induced tetraploids in *Vicia villosa* Roth. Caryologia 63, 292–303.
Väinölä, A., 2000. Polyploidization and early screening of *Rhododendron* hybrids. Euphytica 112, 239–244.
Weiss, D., 2002. Introduction to new cut flowers: domestication of new species and introduction of new trait not found in commercial varieties. In: Vainstein, A. (Ed.), Breeding for Ornamentals: Classical and Molecular Approaches. Kluwer Academic Publishers, Netherlands, pp. 129–137.
Yang, X., Ye, C.-Y., Cheng, Z.-M., Tschaplinski, T.J., Wullschleger, S.D., Yin, W., Xia, X., Tuskan, G.A., 2011. Genomic aspects of research involving polyploid plants. Plant Cell, Tissue and Organ Culture 104, 387–397.

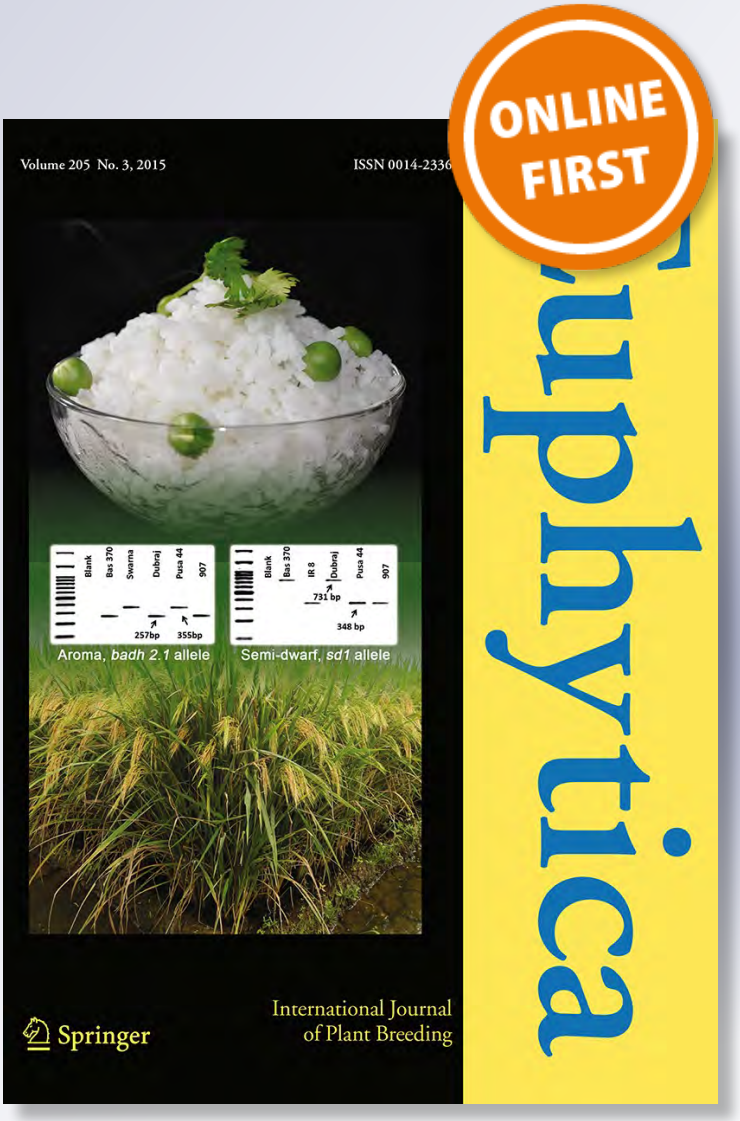
In vitro-induced tetraploids of Plectranthus esculentus *are nematode-tolerant and have enhanced nutritional value*

K. Hannweg, W. Steyn & I. Bertling

Euphytica
International Journal of Plant Breeding

ISSN 0014-2336

Euphytica
DOI 10.1007/s10681-015-1547-4



Springer

Euphytica
DOI 10.1007/s10681-015-1547-4

In vitro-induced tetraploids of *Plectranthus esculentus* are nematode-tolerant and have enhanced nutritional value

K. Hannweg · W. Steyn · I. Bertling

Received: 15 May 2015 / Accepted: 26 August 2015

Abstract *Plectranthus esculentus* (Family: Lamiaceae), or Livingstone potato, is an edible tuberous vegetable which originated in Africa, with central Africa being the centre of origin. *P. esculentus* is found throughout the continent, including the north-eastern regions of South Africa. Although the tubers are edible, limited crop improvement has been achieved; therefore, a study comprising in vitro polyploidisation was carried out with subsequent evaluation of plant nutritional value and nematode tolerance of the induced tetraploids compared with the diploid controls. Tetraploid tubers had a higher starch content compared with the diploids, however there was no significant difference in mineral element content for either the leaves or the tubers when induced tetraploids were compared with the diploid control. Further, induced tetraploids appeared to be significantly more tolerant to rootknot nematode, *Meloidogyne* spp., than the diploids. A significantly higher number of egg masses per root system and number of eggs and J2 (juvenile stage 2) individuals per root system were detected in control plants, compared with tetraploid plants. Induced tetraploidy resulted in plants with a higher nutritional starch concentration and tolerance to rootknot nematode, characteristics which will improve the cultivation and utilisation of the crop. Morphologically, tetraploid plants had fewer, thicker stems per plant compared with diploid plants.

Keywords Livingstone potato · Crop improvement · Chromosome doubling · Polyploid

K. Hannweg (✉) · W. Steyn
Plant Improvement, Agricultural, Research Council – Institute for Tropical and Subtropical Crops, Private Bag X11208, Nelspruit 1200, South Africa
e-mail: Karin@arc.agric.za

K. Hannweg · I. Bertling
Horticultural Science, School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal, Private Bag X01, Scotsville 3209, South Africa

Introduction

Root and tuber crops are globally amongst the most important staple foods. The introduction of crops developed and/or improved using biotechnological methods combined with traditional breeding and selection methods as well as optimum soil fertility and pest management measures could significantly improve yield and crop nutritional value. In general, very little research has been carried out on the nutritional value of edible wild root and tuber species, although indigenous knowledge throughout the world suggests that numerous such species are nutrient-rich and have potential for further development. Moreover, because of their greater tolerance of sub-optimal conditions, the potential of under-utilised wild species to contribute to food security is currently being investigated world-wide. *Plectranthus esculentus,*

Published online: 01 September 2015

the Livingstone potato, originated in central Africa, but is distributed throughout the Continent, including the north-eastern regions of South Africa (Codd 1985). The plant is a perennial shrub of 600–1200 mm in height, with square pubescent stems and subsessile, oppositely-arranged leaves. The edible parts of the plant are the tubers, which are produced by a fibrous root system. The tubers are cylindrical, sometimes branched, 50–100 mm in length and up to 20 mm in diameter. Although there is little information describing cultivation practises, the plant is propagated from sections of stem tubers, usually planted 50 mm to 100 mm deep, on prepared ridges or in beds. After harvesting, tubers used for vegetative propagation can be successfully stored for at least two months under cool, well-ventilated conditions for planting in the following season (Dhliwayo 2002). The species produces tubers even under extremely adverse climatic conditions and therefore contributes to food security in rural areas (Van Wyk and Gericke 2000). Although *P. esculentus* is cultivated throughout Africa as an important vegetable crop, one of the major cultivation challenges experienced by smallholder farmers is a high susceptibility to rootknot nematode (Goodey et al. 1965; Allemann 2002; Xaba and Croeser 2011), made especially more challenging due to the cultivation of other vegetable crops on their lands with consequently high nematode populations in the soil.

A relatively common technique, polyploid induction, has been used in plant breeding since the early 1900's for the development and improvement of a variety of economically-important crops (Gates 1909; Stebbins 1947). Blakeslee and Avery (1937) reported on the first applications in agricultural crops. Such is its importance that a number of reviews have been published in the last decade (Osborn et al. 2003; Soltis et al. 2004; Wendel and Doyle 2005; Chen and Ni 2006; Balao et al. 2011), several highlighting enlargement and induced vigour as well as the enhancement of pest and disease tolerance. Mehta and Swaminathan (1957) and Busey et al. (1993) reported on the higher resistance of polyploids compared with diploids to nematode parasitism. Although polyploidy is a naturally-occurring phenomenon (Soltis and Soltis 2009; Jiao et al. 2011) it can also be artificially induced. Mitotic spindle inhibitors such as colchicine and oryzalin are most widely used to induce chromosome doubling (Dhooghe et al. 2011). Polyploidy has been induced in several species of the Lamiaceae family including basil (Omidbaigi et al. 2010) and lavender (Urwin 2014). Previous studies in our laboratory, and by others (Beltram and Kam 1984; Ramachandran 1982; Ramachandran and Nair 1992; Smith et al. 2004) showed that various plant characteristics of culinary ginger (a rhizomatous species), *Zingiber officinale*, could be improved by inducing polyploidy. Further, several highly-prized and staple root crop species such as potato, sweet potato, turnip, taro, cassava and yam, are natural or induced polyploids (Hilu 1993). Artificial induction of polyploidy therefore provides a means of potentially improving various characteristics of an under-utilised species such as *Plectranthus esculentus*. The aim of this study was, therefore, to induce polyploidy in vitro in *P. esculentus* and subsequently evaluate and compare selected plant characteristics, nematode tolerance, yield and nutritional value of diploid and tetraploid tubers.

Materials and methods

Micropropagation of shoot cultures of *P. esculentus*

In vitro shoot cultures were obtained from the Agricultural Research Council's Vegetable and Ornamental Plant Institute, South Africa. Shoots were subcultured on a 4-weekly basis on Murashige and Skoog (1962) nutrient medium with 3 g l^{-1} Gelrite® and 30 g l^{-1} sucrose added (proliferation medium). No growth regulators were added to the culture medium. All media was autoclaved for 20 min at 121 °C and 1 kPa. Shoot cultures were grown at 25–27 °C with cool white fluorescent light (81 µmol m^{-2} s^{-1}, Phillips 65 W) 27 under a 16:8 h light: dark cycle and shoots rooted spontaneously. Rooted plantlets were washed under running tap water before being planted into Speedling® trays for acclimatisation in a mist-bed at 85 % shading and misting for 5 s every hour for 3 weeks, before being transplanted into larger planting bags containing a 1:1 [v/v] mixture of sand and pine bark. Plants were maintained in a 40 % black shade cloth shadehouse.

Induction of polyploidy

Single nodes were excised from in vitro shoot cultures and subsequently incubated in sterile (autoclaved)

solutions containing 0.01, 0.10, 1.0 and 10.0 g l^{-1} colchicine, either overnight or for 3 days. Two hundred and fifty nodes were used for each treatment. The treated nodes were then cultured on proliferation medium, allowing the axillary buds to grow out and elongate. Once shoots were large enough to be harvested for flow cytometry analysis, usually after 3–4 weeks, samples were collected to verify polyploid induction.

Verification of polyploid induction

Ploidy level of shoots resulting from colchicine treatment was verified using flow cytometry. Samples were prepared for flow cytometry analysis using approximately 0.5 cm^2 leaf tissue. The tissue was macerated with a razor blade in 125 µl nucleus extraction solution (Partec, Germany), after which the homogenate was filtered through a 50 µm mesh filter. The isolated nuclei were stained with 1250 µl of 4′-6-diamidino-2-phenylindole (DAPI) stain (Partec, Germany) prior to commencing flow cytometry analysis using a Partec CYFLOW space flow cytometer. Nuclei isolated from untreated, diploid plantlets were used as a standard. Histograms were analysed using the Partec software package. Shoots confirmed as tetraploid were proliferated on MS medium under the conditions described above. Plantlets were acclimatised as described above. After 12 weeks, plants were transplanted into 20 l potting bags containing a 1:1 [v/v] mixture of pine bark and sand and controlled-release fertiliser granules (Osmocote®).

Morphological characterisation

Morphological characteristics such as plant growth form, number of shoots produced per plant and leaf shape were evaluated and compared between diploid and tetraploid plants.

Nutritional analysis of tubers and leaves

Mature, but not senescing, leaves were harvested from plants and were analysed for macro- and micronutrient content. Tubers were harvested eight months after the acclimatised plantlets were planted into the 20 l potting bags. All analyses were carried out using standard methods (at a South African National Accreditation System- accredited facility) particular to each nutrient with three replicates, twelve plants per replicate for each analysis for both diploids and tetraploids.

Leaf samples were oven-dried at 60 °C for 48 h before being finely milled. Milled samples were extracted overnight using 2:1 nitric acid (55 %), [v/v]: perchloric acid (70 %) solution. Samples were then digested for 6 h at 180 °C on a digestion block before being cooled. Calcium, magnesium, zinc, copper, manganese and iron were determined using atomic absorption spectrophotometry (AOAC Official Method 975.03) whereas potassium and sodium were analysed using flame emission spectroscopy (Varian SpectraAA 250 Plus) according to the method described by Poluektov (1973). Phosphorous and boron were analysed colorimetrically, phosphorous at 660 nm and boron at 430 nm, using an Auto Analyzer (Bran and Luebbe Auto Analyser 3) according to standard methods, Phosphorus reagents, Technicon Industrial Method, method 144-71A (1972) and AOAC Official Method 982.01, respectively.

For nitrogen analysis, samples were oven-dried at 60 °C for 48 h before being extracted for 2 h with concentrated sulphuric acid followed by digestion with hydrogen peroxide. Nitrogen was analysed colorimetrically at 640 nm (ammonia-salicylate complex) using an Auto Analyzer (Bran and Luebbe Auto Analyser 3 (AOAC Official Method 990.02).

The β-carotene content of tubers was analysed using standard methods as described by Horwitz (2000) by the South African Bureau of Standards, Pretoria, South Africa.

Diploid and tetraploid tuber material was analysed for ash, protein, carbohydrate and fat content as well as for vitamins B1 and B2. Amino acid content (serine, aspartic acid, glutamic acid, glycine, threonine, alanine, tyrosine, proline, methionine, valine, phenylalanine, isoleucine, leucine, histidine and lycine) was also determined according to Gehrke et al. (1985).

Ash content was determined by heating the sample at 550 °C overnight. The remaining residue (inorganic matter) was used to determine ash content (AOAC Official Methods 934.01 and 930.15). Protein content of samples was determined using the Kjeldahl method which measures total organic nitrogen (AOAC Official Method 954.01). The organic matter was digested with hot concentrated sulphuric acid and a catalyst mixture added to the acid to raise the boiling point. All nitrogen was converted to ammonia which was measured by titration. Soluble and insoluble carbohydrates were determined by analysing moisture content,

crude protein, ash and fat content with the remainder being total carbohydrates (Greenfield and Southgate 2003). Soluble unbound fat was dissolved in ether at boiling point and evaporated at 105 °C using the Soxtec method (AOAC Official Method 920.39 and its content expressed as a percentage.

The tissue vitamin B1 concentration was determined by derivatising samples to form thiochrome (a highly fluorescent oxidised product of thiamine) after autoclave extraction. A C18 cartridge was used to remove interferences and the vitamins chromatographed by using reversed phase separation as described by Sims and Schoemaker (Sims and Shoemaker 1993). The vitamin B2 concentration was determined after autoclave extraction, centrifugation and dilution. The samples were analysed using reversed phase separation.

Amino acid content was analysed as described by Einarsson et al. (1983) using acid hydrolysis followed by pre-column derivatisation, HPLC separation and detection using a fluorescence detector.

Starch was determined using an iodine-based colorimetric method as described by Xu et al. (1998). Tubers were oven-dried at 65 °C for 48 h for sample preparation and starch content was determined at 620 nm using a WPA-lightwave spectrophotometer.

Nematode studies

Fifteen plants each of diploid control plants and induced tetraploids were evaluated for their tolerance to local *Meloidogyne incognita* race 2 and *M. javanica* populations in a greenhouse experiment. To test the virulence of the nematodes, a highly susceptible tomato (*Solanum lycopersicon* L.) cv. 'Rodade' was used as a reference plant in all experiments. One litre (1 l) black plastic potting bags were filled with steam-pasteurised sandy soil (84 % sand, 14 % silt, 2 % clay and 0.5 % organic matter content). The soil pH (H_2O) was 5.75. Nutrition in the form of Multifeed® Classic (Efekto), was applied as a soil drench every 14 days at a rate of 7.5 g l^{-1} water. One plant was planted per bag. Populations of *M. incognita* race 2 and *M. javanica* were established and maintained on the 'Rodade' tomatoes in a separate greenhouse. Eggs and second stage juveniles (J2) of each of the appropriate species were used to inoculate plants. Inoculation was performed 32 days after planting by pipetting approximately 1000 eggs and J2 of the respective population on exposed roots of each of the seedlings. The roots were covered with soil again after the inoculation.

Fifty-six days after nematode inoculation, the plants were carefully removed from the bags. This period allowed completion of at least one nematode generation (Kleynhans 1991; Fourie 2005). The root systems were rinsed free of adhering soil and debris with running tap water and blotted dry on paper towel. The number of egg masses per root system was counted. Staining of the egg masses to facilitate counting was done by immersion of the root systems in a Phloxine B solution for 20 min (Hussey and Boerma 1981). Root systems were individually inspected and the red-stained egg masses were counted using a stereo microscope. Eggs and J2 were extracted from the root systems using Riekert's (1995) modified NaOCl method. Eggs and J2 were counted and the reproductive potential of each nematode population on each genotype was determined according to Oosterbrink's reproduction factor (Windham and Williams 1988) and calculated as follows: Rf = final egg and J2 numbers (Pf)/initial egg and J2 numbers (Pi).

Statistical analysis

For the polyploid induction/colchicine applications, an appropriate analysis of variance was fitted to the data using PROC GLM procedure of SAS software Version 9.2 of the SAS System for Windows (SAS Institute 2015). Shapiro–Wilk test was performed to test for normality (Shapiro and Wilk 1965) and a Fisher *t* test with least significant difference was calculated at the 5 % significance level to compare treatment means (Ott and Longnecker 2001). For all other experiments, the experimental layout was a complete randomised design (CRD) with 3 replications, twelve measurements per replicate. An analysis of variance was performed at the 5 % significance level using SAS software Version 9.2 of the SAS System for Windows (SAS Institute 2015).

Results and discussion

In vitro proliferation and acclimatisation of *P. esculentus* shoots

Plectranthus esculentus shoots were readily proliferated and spontaneously rooted in vitro. There was no

difference between the multiplication rate (5.0 at a subculture interval of 6 weeks) of diploid and tetraploid plants, although the tetraploid plants did have a tendency for thicker stems and larger leaves in vitro than the diploids. Both diploid and tetraploid plants were readily acclimatised in the mistbed using the methods described, and transplanted into 1 l potting bags 3–4 weeks after acclimatisation.

Verification of polyploid induction of in vitro *shoot cultures P. esculentus using flow cytometry analysis*

Flow cytometry analysis facilitated the identification of pure polyploid plants from the colchicine-treated population containing diploids, mixoploids and polyploids. Polyploidy was induced in in vitro shoots of *Plectranthus esculentus* for all treatments except the 3 day 10 g l^{-1} colchicine treatment where all shoots died. A relatively high percentage of tetraploids was induced and survived for the overnight, 1.0 g l^{-1} (20.40 %), 3 day, 0.1 g l^{-1} (11.20 %) and 3 day, 1.0 g l^{-1} (26.80 %) treatments (Table 1). No octoploids were identified. The interaction of exposure time and colchicine concentration (P value = 0.5501) as well as exposure time and ploidy interaction (P value = 0.9987) were not significant. Further, an investigation of comparison of means for the interaction between concentration and ploidy level was also not significant. However treatment of nodes at 10 g l^{-1} at both exposure times tested appeared to be toxic based on the extremely high mortality of shoots. Earlier studies reported that successful chromosome doubling is dependent on explant type, duration of exposure and concentration of colchicine as well as the genome doubling capacity of the species under investigation (Khosravi et al. 2008; Sun et al. 2009; Dhooghe et al. 2011) and therefore each species requires testing.

Morphological characteristics of polyploid *P. esculentus* plants

In contrast to diploids, induced tetraploid plants showed marked differences amongst various morphological characteristics compared with the diploids (Table 2). Tetraploid plants had, on average, fewer stems than their diploid progenitors (11 stems per plant compared with 24 stems per plant, respectively). Furthermore, stem diameter, was also significantly larger for the induced tetraploids compared with the diploids (5.30 mm compared with 3.29 mm, respectively), although there was no difference in plant height. Leaf index, an indication of leaf shape, was higher for the tetraploids than the diploids, but it was not significant. Leaves of the tetraploid plants were a darker shade of green than the diploids. The alteration of leaf morphology of *P. esculentus* is in line with reports that polyploidisation can alter plant morphology, phenology and physiology (Levin 2002). In a review of polyploidy in plants, Tate et al. (2005) reported on a variety of well-documented studies in which ploidy level alterations can result in higher growth rates, increased secondary metabolite production as well as larger vegetative and reproductive plant parts – particularly in ornamental and food crops.

Table 1 Effect of colchicine exposure time and concentration on polyploidy induction of in vitro shoots of *Plectranthus esculentus*

Treatment (g/l)	2n (%)	2n/4n (%)	4n (%)	4n/8n (%)	8n (%)
Overnight					
0.00	100.0	0.00	0.00	0.00	0.00
0.01	29.60	0.00	2.40	0.00	0.00
0.1	54.80	0.00	7.60	0.00	0.00
1.0	42.00	0.00	20.40	0.00	0.00
10.0	2.80	1.20	0.40	0.00	0.00
3 days					
0.00	100.0	0.00	0.00	0.00	0.00
0.01	90.40	4.80	1.60	0.00	0.00
0.1	8.80	8.80	11.2	4.0	0.00
1.0	26.80	0.00	26.80	0.00	0.00
10.0	0.0	0.0	0.0	0.0	0.0

Data is expressed based on the number of nodes surviving as a percentage of the total number of nodes treated for each exposure time and concentration

Table 2 Comparison of various plant characteristics between diploid and tetraploid *Plectranthus esculentus* plants

	Number of stems/plant	Stem diameter (mm)	Leaf index (breadth/length)
Diploid	24.0b	3.29a	0.41a
Tetraploid	11.0a	5.30b	0.51b

n = 12 each for diploid and tetraploid plants, 3 replicates, $P < 0.05$)

Different letters within columns represent significant differences

Nutritional characteristics of polyploid *P. esculentus* plants

Tuberous crops are cultivated for their starch-rich storage organs which are a rich source of energy in the form of carbohydrates, including starch. There is a dearth of literature describing the impact of induced polyploidy on starch content of tubers. However, most commercial tuber crops such as potato, sweet potato, yam and cassava are all polyploid, many of them being high-yielding triploid varieties (Atherton and Rees 2008) compared with the diploids. Although dry matter content, a characteristic important in the improvement of tuber crops, was not significantly different between the diploid and tetraploid plants (results not shown), tuber starch content of the tetraploid plants was significantly higher than that of the diploids (47.1 % on a dry weight basis compared with 40.0 % for the diploids). Although these figures are lower than that reported previously for *P. esculentus* (Temple et al. 1991), this difference may be ascribed to factors such as cultivation, edaphic conditions or analytical procedures. The starch content still remains significantly higher than potato, 17.1 %, (Potato Board 1980) and sweet potato, average of 23 %, (ARC 1979). β-Carotene, an important antioxidant in tuber crops and precursor of provitamin A, while higher for the tetraploids than the diploids, it was not significantly so, 0.112 mg kg^{-1} compared with 0.07 mg kg^{-1} respectively, under the cultivation conditions of the experimental trial (Table 3). Vitamin A is an essential vitamin and is required for the development and maintenance of eyesight. An extreme deficiency can ultimately lead to the development of blindness. Vitamin A is also required by the body for healing processes after wounding or infection. Polyploidy affects the biosynthesis of a number of metabolites of a variety of biochemical pathways in plants and are generally present in higher concentrations in polyploids than diploids. Several studies involving the mechanism/s thereof have been carried out (Dhawan and Lavania 1996; Zhang et al. 2005; Caruso et al. 2011) although there are few studies describing the effect of polyploidy on β-carotene biosynthesis and levels in induced

Table 3 Comparison between selected nutritional values of tubers harvested from diploid and tetraploid *P. esculentus* plants

Component	Diploid	Tetraploid
Ash (%)	0.64a	0.61a
Protein (%)	0.82a	0.79a
Carbohydrates (%)	21.78a	18.99a
Fat (%)	0.198a	0.268a
Starch (g/kg)	400a	470b
β-Carotene (mg/kg)	0.07a	0.112a
Vitamin B1 (mg/100 g)	0.01a	0.01a
Vitamin B2 (mg/100 g)	0.01a	0.01a
Alanine (g/100 g)	0.042a	0.036a
Aspartic acid (g/100 g)	0.056a	0.050a
Glutamic acid (g/100 g)	0.07a	0.06a
Histidine (g/100 g)	0.04a	0.056a
Isoleucine (g/100 g)	0.032a	0.032a
Leucine (g/100 g)	0.034a	0.032a
Lysine (g/100 g)	0.038a	0.034a
Methionine (g/100 g)	0.01a	0.01a
Phenylalanine (g/100 g)	0.036a	0.032a
Proline (g/100 g)	0.028a	0.022a
Serine (g/100 g)	0.034a	0.032a
Threonine (g/100 g)	0.046a	0.042a
Tyrosine (g/100 g)	0.022a	0.028a
Valine (g/100 g)	0.038a	0.036a

n = 12 each for diploid and tetraploid plants, 3 replicates, $P < 0.05$)

The same letter across rows indicates no significant difference. (results are expressed as fresh weight)

Table 4 Comparison between selected mineral values of leaves and tubers harvested from diploid and tetraploid *P. esculentus* plants

Component	Diploid	Tetraploid
Leaves		
N (%)	2.65a	3.10a
P (%)	0.154a	0.153a
K (%)	2.01a	2.23a
Ca (%)	1.11a	1.00a
Mg (%)	0.554a	0.546a
Zn (mg/kg)	46.75a	41.75a
Cu (mg/kg)	9.00a	8.33a
Mn (mg/kg)	804a	714.14a
Fe (mg/kg)	94.91a	91.63a
B (mg/kg)	32.5a	19.7a
Tubers		
N (%)	0.59a	0.63a
P (%)	0.122a	0.125a
K (%)	1.22a	1.14a
Ca (%)	0.10a	0.08a
Mg (%)	0.149a	0.141a
Zn (mg/kg)	17.00a	17.40a
Cu (mg/kg)	3.40a	4.00a
Mn (mg/kg)	16.60a	17.5a
Fe (mg/kg)	34.50a	34.75a
B (mg/kg)	17.40a	18.8a
Na (%)	0.0238a	0.0212a

$n = 12$ each for diploid and tetraploid plants, 3 replicates, $P < 0.05$)

The same letter across rows indicates no significant difference

Results are presented as per dry mass

polyploids. Jaskani et al. (2005), however, reported that β-carotene content was higher in induced tetraploids of watermelon compared with the diploids. There were no significant differences between diploids and tetraploids in terms of any of the other macro- and micronutrients evaluated (Table 4), but this, together with the effect of polyploidy on β-carotene, needs to be investigated further under in-field cultivation conditions which are sub-optimal compared with a pot trial.

Tolerance of polyploid plants to nematodes

One of the most significant biotic factors affecting any tuber crop is nematode infestation. Nematodes cause significant yield losses and applications of both chemical and environmentally-friendly products to reduce the risk of damage to crops is an industry worth millions of US dollars. Management strategies, as well as breeding and selection of more tolerant varieties, is critical for any tuber crop industry (Jones et al. 2011). *Plectranthus esculentus* is highly susceptible to root-knot nematodes (*Meloidogyne* spp.). Tetraploid and diploid plants were challenged with rootknot nematodes in a pot experiment to determine if polyploidy resulted in any degree of tolerance. The results of the trial showed that tetraploid plants were significantly more tolerant to rootknot nematodes (Table 5). There was a significant difference in the number of egg masses per root system for the diploids compared with the tetraploids. Tetraploid plants had a significantly lower egg mass number per root system compared with the diploids (7.9 egg masses/root system compared with 28 egg masses/root system, respectively). Tetraploids were therefore found to be moderately resistant to rootknot nematodes compared with the diploids which were highly susceptible according to the classification system of Murray et al. (1986). Similarly, the number of eggs and J2 per root system was also significantly lower for the tetraploids compared with the diploids, 3196 compared with 12,187, respectively). The reproduction factor (Rf-value) is an indication of tolerance to nematode infestation, the lower the Rf-value the more tolerant the plant species is to nematode attack. Tetraploids had a significantly lower Rf-value, 3.17, compared with the diploids, 12.15. Although there was a significant improvement in nematode tolerance over the diploids, host status is considered good compared with diploids with a host status defined as excellent according to the Rf classification system described by Windham and Williams (1988). In this study, the tolerance of tetraploids compared with diploids to rootknot nematode was investigated and examination of tuber anatomy at light microscope level did not reveal any fundamental differences between the induced tetraploids and diploids prior to nematode challenging, nor after challenging, possibly due to the young plant age. However, future histopathological studies may reveal symptoms of nematode infestation such as feeding sites and associated changes in cell structure on more established plants. In potato, rootknot nematode damage to tubers which is characterised not only by losses in yield, but damage can manifest as blistering

Table 5 Comparison of number of egg masses per root system, number of eggs and J2 per root system and reproduction factor value for diploid and tetraploid *Plectranthus esculentus* plants inoculated with J2 and eggs of *Meloidogyne* species

	Number of egg masses per root system	Number of eggs and J2 per root system	Reproduction factor value (Rf-value)
Diploid	28b	12187b	12.15b
Tetraploid	7.9a	3196a	3.17a

n = 15 each for diploid and tetraploid plants, 3 replicates ($P < 0.05$)

Different letters within columns represent significant differences

on the surface of tubers as well as internal browning (Volvlas et al. 2005). Furthermore, Jatala et al. (1982) reported that externally-visible symptoms may only develop after a period of storage after internal symptoms have developed on potato. This manifestation of tuber damage is critical in terms of *P. esculentus* tubers which are commonly stored for relatively long periods of time before consumption or re-establishment (van Wyk and Gericke 2000) and this should be investigated further. Moreover, there is the possibility that growers unwittingly facilitate the spread of nematodes using diseased seed tubers which may not yet exhibit symptoms externally.

Future prospects regarding improvement

Almost no breeding and selection or improvement has been carried out on *Plectranthus esculentus*. This is the first report on the improvement of this species using artificial polyploid induction. Furthermore, it is also one of the few published reports on the induction of nematode tolerance in induced polyploid plants. Although the histopathological response/s and manifestation of symptoms of infestation as well as the mechanism of tolerance to nematodes by the tetraploid plants still needs to be elucidated, polyploid induction could provide a valuable tool in improving nematode-tolerance in a number of crops; however, the effect of polyploidy on other critical horticultural characteristics such as yield and crop quality would need to be taken into account and investigated. Physiological investigations i.e. assimilation and water stress studies are being explored further after preliminary investigations revealed that the tetraploids appeared to have higher assimilation rates and superior water stress tolerance than the diploids.

Acknowledgments The authors would like to thank the Agricultural Research Council and Department of Science and Technology (ECS Programme) for financial assistance. Mr Gerrit Visser and Ms Elsabe Aylward of the ARC-Institute for Tropical and Subtropical Crops are thanked for technical assistance for the flow cytometry and mineral nutrition samples, respectively, as well as ARC-Irene Analytical Services for the nutritional analyses. Mardé Booyse of the ARC Biometry Unit is thanked for assistance with the statistical analysis.

References

Allemann J (2002) Evaluation of *Plectranthus esculentus* as a potential vegetable crop. Ph.D. Dissertation, University of Pretoria, South Africa

ARC (1979) Annual sweet potato research report. ARC-Roodeplaat Vegetable and Ornamental Plant Institute, Pretoria

Atherton J, Rees A (eds) (2008) Tropical root and tuber crops: cassava, sweet potato, yams and aroids, vol 17., Crop protection science in horticulture, CAB International, Wallingford

Balao F, Herrera J, Talavera S (2011) Phenotypic consequences of polyploidy and genome size at the microevolutionary scale: a multivariate morphological approach. New Phytol 192:256–265

Beltram I, Kam Y (1984) Cyto taxonomic studies in the Zingiberiaceae. Notes R Bot Garden, Edinb 41:541–557

Blakeslee A, Avery A (1937) Colchicine and double diploids. J Hered 28:411–412

Busey P, Giblin-Davis R, Center B (1993) Resistance in Stenotaphrum to the sting nematode. Crop Sci 33:1066–1070

Caruso I, Lepore L, de Tommasi N, dal Piaz F, Frusciante L, Aversan R, Garramone R, Carputo D (2011) Secondary metabolite profile in induced tetraploids of wild *Solanum commersonii* Dun. Euphytica 8:2226–2237

Chen Z, Ni Z (2006) Mechanisms of genomic arrangements and gene expression changes in plant polyploids. BioEssays 28:240–252

Codd L (1985) The genus *Plectranthus*. Flora S Afr 28:137–172

Dhawan O, Lavania U (1996) Enhancing the productivity of secondary metabolites via induced polyploidy: a review. Euphytica 87:81–89

Dhliwayo P (2002) Underexploited tuber crops in Zimbabwe: a study on the production of Livingstone Potato (*Plectranthus esculentus*). PGR Newsletter, FAO-Bioversity 130:77–80

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissues in vitro. Plant Cell Tissue Organ Cult 104:359–373

Einarsson S, Josefsson B, Lagerkvist S (1983) Determination of amino acids with 9-fluorenylmetahyl chloroformate and reversed-phase high-performance liquid chromatography. J Chromatogr 282:609–618

Fourie H (2005) In vivo evaluation of resistance to *Meloidogyne incognita* race 2 (Nematoda: Tylenchida) and identification of genetic markers for this trait in soybean (Glycine max). Ph.D. Thesis. Catholic University of Leuven, Belgium

Gates R (1909) The stature and chromosomes of *Oenanthera gigas* De Vries. Arch für Zellf 3:525–552

Gehrke C, Wall L, Absheer J, Kaiser F, Zumwalt R (1985) Sample preparation for chromatography of amino acids: acid hydrolysis of proteins. J Assoc Off Analyt Chem 68:811–821

Goodey J, Franklin M, Hooper D (1965) The nematode parasites of plants catalogued under their hosts. Commonwealth Agricultural Bureaux, Farnham Royal: Bucks, p 214

Greenfield H, Southgate D (2003) Food composition data—production, management and use, 2nd edn. Food and Agricultural Organization of the United Nations, Rome

Hilu K (1993) Polyploidy and the evolution of domesticated plants. Am J Bot 80:1494–1499

Horwitz W (2000) Official methods of analysis of AOAC International, 17th edn. AOAC International, Gaithersburg

Hussey R, Boerma H (1981) A greenhouse screening procedure for root-knot nematode resistance in soybeans. Crop Sci 21:794–796

Jaskani M, Kwon S, Kim D (2005) Comparative study on vegetative, reproductive and qualitative traits of seven diploid and tetraploid watermelon lines. Euphytica 145:259–268

Jatala P, Booth R, Wiersema S (1982) Development of *Meloidogyne incognita* in stored potato tubers. J Nematol 14:142–143

Jiao Y, Wickett N, Ayyampalayam S, Chanderbali A, Landherr L, Ralph P et al (2011) Ancestral polyploidy in seed plants and angiosperms. Nature 473:97–100

Jones J et al (2011) Genomics and molecular genetics of plant-nematode interactions:Chapter 2. Springer Science + Business Media B.V, New York, pp 21–43

Khosravi P, Jafarkhani Kermani M, Nematzade G, Bihamta M (2008) Role of mitotic inhibitors and genotype on chromosome doubling of Rosa. Euphytica 160:267–275

Kleynhans K (1991) The root-knot nematodes of South Africa. Technical Communication No. 231. Pretoria: Department of Agricultural Development, 61 pp

Levin D (2002) The role of chromosomal change in plant evolution. Oxford University Press, Oxford

Mehta R, Swaminathan M (1957) Studies on induced polyploidy in forage crops. Indian J Genet Plant Breed 17:27–57

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Murray J, Poole T, Ostazeski S (1986) Techniques for determining reproduction of *Meloidogyne gramnis* on Zoysiagrass and Bermudagrass. Plant Dis 70:559–560

Omidbaigi R, Mirzaee M, Hassani M, Sedghi Moghadam M (2010) Induction and identification of polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment. Int J Plant Prod 4:87–98

Osborn T, Chris Pires J, Birchler J, Auger D, Jeffery Chen Z, Lee H-S, Comai L, Mablung A, Doerge R, Colot V et al (2003) Understanding mechanisms of novel gene expression in polyploids. Trend Genet 19:141–147

Ott R, Longnecker M (2001) An introduction to statistical methods and data analysis. 5th edn, Duxbury Press, Belmont p 440 (pp 1–1152) t test and statistical principals

Poluektov N (1973) Analytical Methods of Flame Photometry. Technika knjiga, Belagrade

Potato Board (1980) Nutritive value of potatoes. Fmg S Afr Potatoes A2

Ramachandran K (1982) Polyploidy induced in ginger by colchicine treatment. Curr Sci 51:288–289

Ramachandran K, Nair P (1992) Cytological studies on diploid and autotetraploid ginger (*Zingiber officinale* Rosc.). J Spices Aromat Crop 1:125–130

Riekert F (1995) An adapted method for extraction of root-knot nematodes from maize root samples. Afr Plant Prot 1:41–43

SAS Institute (2015) The Statistical Procedure manual. North Carolina 27513: SAS Campus Drive, Cary

Shapiro S, Wilk M (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611

Sims A, Shoemaker D (1993) Simultaneous liquid chromatographic determination of thiamine and riboflavin in selected foods. J AOAC Int 76:1156–1160

Smith M, Hamill S, Gogel B, Severn-Ellis A (2004) Ginger (*Zingiber officinale*) autotetraploids with improved processing quality produced by an in vitro colchicine treatment. Aust J Exp Agric 44:1065–1072

Soltis P, Soltis D (2009) The role of hybridization in plant speciation. Annu Rev Plant Biol 60:561–588

Soltis D, Soltis P, Tate J (2004) Advances in the study of polyploidy since plant speciation. New Phytol 161:173–191

Stebbins G (1947) Types of polyploidy: their classification and significance. Adv Genet 1:403–429

Sun Q, Sun S, Li L, Bell R (2009) In vitro colchicine-induced polyploidy plantlet production and regeneration from leaf explants of the diploid pear (*Pyrus communis* L.) cultivar 'Fertility'. J Hortic Sci Biotechnol 84:548–552

Tate J, Soltis D, Soltis P (2005) Polyploidy in plants. The evolution of the genome. Elsevier Inc., Burlington, pp 371–426

Temple VJ, Ojobe TO, Onobun CE (1991) Chemical composition of Livingstone potato tubers (*Plectranthus esculentus*). J Sci Food Agric 56:215–217

Urwin N (2014) Generation and characterisation of colchicine-induced polyploid *Lavandula* x *intermedia*. Euphytica 197:331–339

Van Wyk B-E, Gericke N (2000) People's Plants: a guide to useful plants of southern Africa. Briza Publications, Pretoria, p 351

Volvlas N, Mifsud D, Landa B, Castillo P (2005) Pathogenicity of the root-knot nematode *Meloidogyne javanica* on potato. Plant Pathol 54:657–664

Wendel J, Doyle J (2005) Polyploidy and evolution in plants. In: Henry R (ed) Plant diversity and evolution: Genotypic and phenotypic variation in higher plants. CAB International, Wallingford

Windham G, Williams W (1988) Reproduction of *Meloidogyne javanica* on corn hybrids and inbreds. Ann App Nematol 2:25–28

Xaba P, Croeser P (2011) Wild Potato. Veld and Flora 2011:180-181

Xu C, Chen W, Chen K, Zhang S (1998) A simple method for determining the content of starch–iodine colorimetry. Biotech 8:41–43

Zhang A, Chang L, Xue J (2005) Progress in research on inducing the polyploid of medicinal plants. Zhongguo Zhong Yao Za Zhi 30:645–649