THE POTENTIAL OF THIDIAZURON IN *IN-VITRO* PROPAGATION OF SELECTED ORNAMENTAL PLANTS.

BY

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of science in Horticulture.

FACULTY OF AGRICULTURE

COLLEGE OF AGRICULTURE AND VETERINARY SCIENCES

UNIVERSITY OF NAIROBI

2004

DECLARATION

This thesis is my original work and has not been presented

For a degree in any university.

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This thesis has been submitted for examination with our approval as the

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DEDICATION

To my late uncle, Ernest Irangi and late sister Dr Beatrice Onamu, who inspired me to further my education in Agricultural sciences. My parents George Onamu and Arzibether Onamu for their encouragement throughout my studies. My dear son Ernest Mwasicho. Learning never ends and there is need for faith in science too at times.

AKNOWLEDGEMENTS

The author wishes to thank her supervisors, Dr. Hutchinson and Dr. Obukosia for their guidance, encouragement and criticism throughout the entire period of the study and preparation of the thesis.

The author also wishes to thank Mbugua farm at Tigoni, Limuru for providing tuberose and *Ornithogalum* plants used in the study. Appreciation is also extended to Mr. Njenga of KARI, Floriculture project, for providing the *Alstroemeria* plants and locating Mrs Margaret Wamboi, a small-scale flower farmer who provided the carnation plants.

The author also wishes to thank the Prof. Imungi, the Dean of agriculture for providing Thidiazuron. Not forgetting Dr Wamocho of JKUCAT for supplying additional thidiazuron. Last but not least, the author wishes to appreciate the collaborative effort provided by technicians in plant pathology, soil science (microbiology), food science and biotechnology laboratories at the University of Nairobi.

The author feels indebted to scientist and library staff at ILRI, CIP, KARI (HQT) and KEPHIS for assisting in literature review and photography. Finally, the author wishes to thank the University of Nairobi for awarding her a scholarship without which it was not going to be possible to carry out the study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

BA	Benzyladenine
BAP	6-Benzylaminopurine
CIP	International Potato Center
FPEAK	Fresh Produce Exporters Association of Kenya
HCDA	Horticultural Crops Development Authority
ILRI	International Livestock Research Institute
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KARI	Kenya Agricultural Research Institute
KEPHIS	Kenya Plant Health Inspectorate Service
KIN	6-Furfurylaminopurine
MSO	Murashige and Skoog medium without plant growth regulators
PGRS	Plant Growth Regulators
NAA	Alpha-Naphthalene-acetic acid
TDZ	N'-phenyl-N'-1,2,3-thidiazol-5-ylurea; thidiazuron
2iP	Isopentinyl adenine

ABSTRACT

ABSTRACT

The potential of the phenylurea derivative, (N'-phenyl-N'-1,2,3-thidiazol-5-ylurea, thidiazuron, TDZ) in stimulating morphogenic responses from shoot-tip cultures of various ornamental crops (*Polianthes tuberosa* L., *Alstroemeria aurantiaca* cv. Rosita, *Ornithogalum saundersiae*, and *Dianthus caryophillus* L. cv. Yair), when compared with a combination of auxin and cytokinin was investigated. Whole plant regeneration was achieved from shoot-tip explants in all the crops tested either directly or indirectly (via an intervening callus phase). In carnation, *Ornithogalum*, and tuberose, TDZ induced morphogenic responses with an efficacy higher than a combination of KIN+NAA, BA+NAA, and BAP+NAA, respectively. TDZ promoted a comparable morphogenic response to that of a combination of BAP and NAA in *Alstroemeria*. In addition, the effect of various concentrations of TDZ (0.1, 0.4, 1.0, and 5.0μM), applied for various durations (3, 10, 24, and >40 days) was determined in carnation shoot-tip cultures. Higher TDZ concentration (5.0μM), compared to 0.1, 0.4 and 1.0μM applied for a short duration (3 days) was found to be optimum culture conditions for the *in- vitro* regeneration of plantlets. The ability of TDZ to substitute for auxin and cytokinin requirement, further suggests its role in modulating endogenous growth substances.

Overall, thidiazuron was more potent or equally potent in inducing morphogenic responses *in -vitro* in the selected ornamental crops.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the horticulture industry in Kenya

During the past decade, horticulture has been the fourth largest foreign exchange earner in Kenya, with total exports valued at Kshs 13 billion in 1996 (HCDA, 1997). Rescently, horticulture has overtaken coffee to become Kenya's second largest export earner after tea (Madeley, 1998). This is because of marketing problems which coffee has been experiencing in the past few years. Floriculture contributes the highest value: volume ratio within the fresh produce sector of the horticultural industry (Hutchinson, 1997) and therefore plays a significant role in the export market. However, the local market is relatively insignificant and is limited to urban center offices and special occasions such as weddings and funerals. Flower production provides diversification and a potentially high profit cropping alternative. Floriculture is labour intensive (Hutchinson, 1997) and the income per unit area of input is high compared to other agricultural produce (KARI, 1993). It is one of the areas, which backs the governments' efforts in creating employment, improving the living standards of farmers and the Kenyan economy as a whole (Hutchinson, 1997; Ouko, 1997). The major floricultural crops produced for the local and export markets are cut flowers, which include roses, carnations, *Alstroemeria*, and statice (Hutchinson, 1997).

Kenya exports her cut-flowers mainly to the Netherlands (64.4%), Germany (19%), United Kingdom (9.9%), and Switzerland (3.4%) (HCDA, 1994; Mutui, 1999). Kenya however faces stiff competition from Israel, Spain, Zimbabwe, Zambia,

South Africa, Thailand, Malawi, Tanzania and other developing countries, for the European market (Gray, 1996).

In order to compete successfully, high standards on the quality and quantity of cut-flowers have to be maintained (Madeley, 1998). In addition to the stiff competition mentioned above, the floriculture industry in Kenya experiences a number of problems, which include: lack of adequate clean planting materials, poor quality seed, lack of new varieties, unavailability of cold storage facilities, lack of reliable marketing channels, pests and diseases, limited irrigation facilities and inconsistent research, especially in post-harvest technologies and micropropagation techniques (Wabule *et al.*, 1991).

1.2 Problem definition

Low yield, poor quality flowers, and high cost of production are problems associated with production of cut-flowers in Kenya (HCDA, 1997; KARI, 1993). Lack of adequate clean planting materials highly contributes to these problems (Hutchinson, 1998; Yako, 1999). As a result, large-scale farmers import clean planting materials, which has proved to be very expensive due to the high charges on breeder's rights and royalties (Mutui, 1999). In addition, many small and medium-scale farmers use the previous season's crop as a source of propagules, and traditional vegetative propagation methods. This system has the disadvantage of transmitting diseases from parents to propagules, and a slow rate of multiplication (Hartmann and Kester, 1986; Wang *et al.*, 1999). To compound the problem, many ornamental plants produce sterile seeds or seeds with poor germination performance. Besides, propagation by seed leads to segregation of desired genetic characteristics (Wang *et al.*, 1999; Hussey, 1983). Therefore, there is need to produce adequate clean planting materials, through the development of more efficient propagation methods and the

regeneration of plants through *in-vitro* propagation methods. Meristem (shoot-tip) cultures have been used in several plants for rapid clonal propagation of clean plant materials. Reports on TDZ mediated regeneration systems, hardly used shoot-tip explants as initial explants.

Few protocols have been developed based on the Skoog and Miller theory (1957). As a result, there has been a slow development of efficient protocols, which in turn has hampered the utilization of micropropagation techniques (Kitto, 1997). Despite numerous reports on more efficient protocols using TDZ compared to a combination of auxin and cytokinin in many plant systems, limited reports are available for many ornamental plants.

In the present study, a number of economically important ornamental plants in Kenya were selected, which include: *Polianthes tuberosa* L., *Alstroemeria* cv Rosita, *Dianthus caryophillus* L. cv Yair, and *Ornithogalum saundersiae*

1.3 Justification

Cut flower production contributes the highest value: volume ratio within the fresh produce sector of the horticultural industry (Hutchinson, 1997). In addition, the floriculture industry creates employment, improving the living standards of farmers (Ouko, 1997; Hutchinson, 1997). The ornamental crops used in the present study are widely grown by small to medium scale farmers. This is due to their minimum management requirements, and ability to grow successfully under out door conditions (Chebet, 1999; Mutui, 1999). The expansion of the floriculture industry in Kenya is hampered by the lack of adequate clean planting materials and the development of new varieties among other problems (Wabule *et al.*, 1991).

There is need to develop efficient *in-vitro* regeneration protocols in order to fully utilize micropropagation techniques (Kitto, 1997). This can be achieved through

the manipulation of regeneration pathways, type of explant, nutrient salts, culture conditions, and plant growth regulators. The manipulation of PGRs has proved to be a faster, cheaper, easy and simple method compared to methods involving the manipulation of regeneration parthways, culture environment, and nutrient salts (Hartmann and Kester, 1986). Traditionally, a combination of auxin and cytokinin have been used to stimulate morphogenic responses under *in-vitro* conditions, based on the Skoog and Miller (1957) hypothesis, which states that morphogenic responses in plant tissue cultures are regulated primarily by an intricate balance of phytohormones, mainly auxins and cytokinins.

In the present study, TDZ, a potent plant growth regulator (Murthy *et al.*, 1998; Hutchinson *et al.*, 1996a; Visser *et al.*, 1992), was used to study its induction of morphogenic responses in selected ornamental plants. TDZ induces morphogenic responses with an efficiency comparable to or higher than that reported for other adenine containing cytokinins (Murthy *et al.*, 1998), and a combination of auxin and cytokinin (Hutchinson et al., 1996: Visser et al., 1992). In addition, much lower concentrations have been used for short duration of exposure (Visser *et al.*, 1992; Preece, 1991).

TDZ has been reported to act directly as a cytokinin, and or through the modulation of endogenous plant growth substances to induce morphogenic responses (Hutchinson *et al.*, 1996a; Murthy *et al.*, 1998).

A few authors have attempted to use TDZ in stimulating shoot proliferation in carnations cv 'white Sims'(Watad *et al.*, 1996; Zuker *et al.*, 1995). However, they used different varieties from those grown under Kenyan conditions and stem segments as initial explants. In both cases, some important issues were not addressed, they include: optimum level of TDZ to be used in regeneration of shoots and the

duration of exposure to TDZ in carnation cv. Yair shoot-tip cultures, which are widely grown under Kenyan conditions by both small and large scale farmers (KARI, 1998; HCDA, 1996).

Lin *et al.* (1997) developed an efficient protocol for regeneration of shoots from leaf explants of VV24 genotype (Van Stavereen of Netherlands). In the regeneration procedure, they used IBA and TDZ to induce shoots and BAP at 2.2μM for regeneration of shoots. In the present study, the effect of TDZ alone or in combination with NAA on shoot regeneration in Alstroemeria cv Rosita commonly grown in Kenya using shoot-tip explants as initial material was determined.

Tuberose and *Ornithogalum* have increasingly become important cut flowers in Kenya (HCDA, 1995; Mutui, 1999), unfortunately, limited studies on *in-vitro* propagation have resulted in the potential of micropropagation techniques not being fully realised in these crops, hence the numerous problems still affecting the crops. Based on the present literature review, no reports on TDZ mediated morphogenic responses in tuberose and *Ornithogalum* were accessed.

In the current study, emphasis was placed on the use of shoot-tip explants because the problem of inadequate clean planting materials was reported as contributing highly to low yields, poor quality and high cost of production in the floriculture industry (Yako, 1999). Meristem (shoot-tip) culture is a commonly used *in-vitro* method for clonal propagation and for virus eradication in *Allium* species (Rabinowitch and Brewster, 1990), carnations (Besemer, 1980), *Alstroemeria* (Bridgen *et al.*, 1990), *Solanum tuberosum* L. (Dodds, 1989). This method maintains high genetic stability and consequently, the isolated meristem-tips can be used for *in - vitro* germplasm conservation (Rabinowitch and Brewster, 1990). Shoot-tip culture

has also been used for experimental mutagenesis and polyploidy breeding with much success than intact plant or callus in Allium species (Novak, 1983; Novak, 1977).

1.4 Objectives

The overall objective of this study is to determine the potential of Thidiazuron in stimulating morphogenic responses in ornamental plant cultures of *Alstroemeria,* Carnations, Tuberose, and *Ornithogalum*, in comparison to the conventional combinations of auxin (NAA) and cytokinin (Kinetin and BAP) in the growth media.

The specific objectives were

1. Investigate the effect of TDZ compared to a combination of auxin and cytokinin on regeneration of plantlets from shoot-tip explants of tuberose, *Alstroemeria*, *Ornithogalum,* and carnation.

2. Investigate the effect of dose and duration of exposure to TDZ on regeneration of plantlets in carnation shoot tip cultures.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Introduction

Cut-flower production in Kenya has become increasingly important as a source of employment and foreign exchange earner (HCDA, 1997; Hutchinson, 1997; Madeley, 1998). In the present study, *Polianthes tuberosa* L. (Tuberose), *Alstroemeria* cv. Rosita, *Ornithogalum saundersiae*, and *Dianthus caryophyllus* cv. Yair (Carnation) were used. These ornamental plants are widely grown in Kenya by both small to medium scale farmers, mainly due to their high productivity, ease of management (including out door planting), and high market demand (Wabule *et al.*, 1991).

Traditionally, cut-flowers have been vegetatively propagated using cuttings, rhizomes, and bulbs (Hartmann and Kester, 1985). The draw back with this method of propagation is the transmission of diseases, especially viruses, loss of vigor and productivity (Besimar, 1980; Wang *et al.*, 1999). The use of seeds has not been very successful, since many ornamental plants produce sterile seeds, or seeds with poor germination performance. Furthermore, propagation by seed leads to segregation of desired genetic characteristics (Wang *et al.*, 1999).

Tissue culture techniques have several advantages over conventional propagation methods (Hussey, 1983). In combination with conventional ornamental crop improvement programmes, *In vitro* techniques could prove useful for:

1. Mass clonal propagation of selected genotypes
2. Production of disease-free clones
3. Germplasm conservation and

4. Development of new varieties via cellular or molecular genetics.

However, the advantages mentioned above have not been fully realised due to a number of constraints. They include; requirements for specialised facilities and advanced skills for their operation (Hartmanm and Kester, 1985; Kitto, 1997), labour intensive methods, as a result, the cost of propagation is relatively high, thereby only feasible for high value crops (cut-flowers) and/or in areas where labour is relatively cheap (developing countries). In addition, efficient protocols for proliferation, rooting, acclimation and re-establishment have not been fully developed for most horticultural crops (Kitto, 1997). Besides, literature on *in vitro* propagation of tropical ornamental plants (Tuberose and *Ornithogalum* spp.) is scarce as a result of few studies conducted on these plants.

In this study, emphasis is placed on *in vitro* propagation of shoot-tip explants from selected ornamental plants. The potential of thidiazuron, a plant growth regulator compared to a combination of auxin and cytokinin is investigated.

2.2 *In-vitro* Morphogenesis

In vitro plant morphogenic responses can be achieved through somatic organogenesis and somatic embryogenesis (Hartmanm and Kester, 1985; Wang *et al.*, 1999). These responses can be achieved directly or indirectly through callus formation from the initial explant material (Hartmanm and Kester, 1985). Conventionally, morphogenic responses have been induced using a combination of auxins and cytokinins based on the Skoog and Miller medium formulation (1957).

Callogenesis

Callogenesis is the formation of callus, which consists of a group of undifferentiated cells (Hartmanm and Kester, 1985; Salisbury and Ross, 1991). This group of undifferentiated cells represents the first stage in morphogenic responses.

Callus induction has been achieved in the presence of exogenous auxins. Cytokinins in nutrient media are not usually necessary and only when they are in combination with auxins will callus formation result (Novak, 1990; Skoog, 1980). Callus cells can be induced to develop into shoots in medium containing a high cytokinin: auxin ratio and or roots in medium with a low cytokinin:auxin ratio, through somatic organogenesis (Novak, 1990; Salisbury and Ross, 1991).

Organogenesis

Organogenesis is the regeneration of plant organs (roots, shoots, and bulblets) or tissues from explants. This process can be direct or indirect through the mediation of callus. Sanyal et al. (1998) observed shoot regeneration and the formation of buds from leaf callus tissue in medium containing low concentrations of NAA (0.2-0.5mg L^{-1}) in combination with 2.0mg L^{-1} BA in *Polianthes tuberosa* L. There is no information on regeneration of shoots from shoot-tip explants of *Polianthes tuberosa* L. using an auxin and cytokinin supplemented in the medium.

In *Alstroemeria* spp., a combination of BAP+NAA has been used to induce shoot regeneration. Bridgen *et al.* (1992), demonstrated the multiplication of rhizomes in *Alstroemeria* hybrids using a combination of 1-5mg L^{-1} BAP and 0.01mg L^{-1} NAA. Similarly, Gabrezuesker (1995), used a combination of 2mg L^{-1} BAP and 0.5mg L^{-1} NAA to regenerate shoots from shoot-tip explants of an .*Alstroemeria* spp.

Nayak and Sen (1995), demonstrated a rapid and stable propagation of *Ornithogalum umbellatum* L. shoots in long-term callus cultures, or directly from scale explants using a combination of 0.5mg/l BA and 2mg/l NAA. Previously, a combination of KIN and NAA at 0.2mg L^{-1} KIN and 0.2mg L^{-1} NAA (George and Sherrington, 1984; Zimmerman *et al.*, 1986), was used in the establishment phase for the regeneration of shoots from carnation cv. White Sim shoot-tip explants. Kyte

(1990), used a higher KIN concentration (2.0mg L^{-1}) during the establishment phase. Likewise, Mujib and Pal (1994), obtained optimum shoot regeneration with a combination of 0.2mg L^{-1} NAA and 0.5mg L^{-1} KIN in carnation cv. William Sim shoot-tip and nodal explants. Apart from somatic organogenesis, shoot regeneration is also achieved through somatic embryogenesis.

Somatic embryogenesis

Somatic embryogenesis is an orderly progression of development by which somatic cells undergo a developmental sequence similar to that seen in zygotic embryos, leading to the differentiation of complete plants. This developmental process is believed to be regulated by an intricate balance of phytohormones, mainly auxins and cytokinins (Komamine *et al.*, 1992). Somatic embryos can be from single or multiple cells. Plantlets from such cells regenerate faster, are more uniform and with less genetic aberrations than those regenerated through callus induction. In a wide variety of species that produce embryogenic cultures, auxins namely 2,4-D and NAA, alone or in combination with cytokinins have been used in the induction and proliferation of somatic embryos (Atanassov and Brown, 1984; Brown, 1988; Stuart and Strickland, 1984). In geranium, a combination of auxin and cytokinin at 1.0μM IAA and 8.0μM BAP was shown to induce somatic embryogenesis (Marsolais *et al* ., 1991; Vissser *et al.*, 1992; Hutchinson *et al.*, 1996a). In a tetraploid *Alstroemeria* cultivar (A. *pelegrina* x A. *psittacina*), indirect somatic embryogenesis was achieved using a combination of 10 or 20μM NAA and 10 or 20μM KIN from zygotic embryo callus derived cell suspension cultures (Hutchinson *et al.*, 1997). Both direct and indirect somatic embryogenesis has been demonstrated in carnation using a combination of auxin and cytokinin. Yantcheva *et al.* (1998), demonstrated conditions for efficient direct somatic embryogenesis and plant regeneration from leaf explants

of carnation cultivars Lena (SIM group) and Bulgarian spray cultivars. They included a combination of 1mg L^{-1} 2,4-D and 0.2mg L^{-1} BAP in liquid MS medium, for the induction of embryoids without an additional callus phase. Indirect somatic embryogenesis was reported using internodal callus initiated by 3μM 2,4-D and transferred to medium without PGRs (Frey *et al.*, 1992). Similarly, Sankhla *et al.* (1995), achieved indirect somatic embryogenesis from carnation cv. German Red petal explants using a combination of BAP and NAA.

2.3 *In- vitro* morphogenetic Responses to Thidiazuron

Thidiazuron

Thidiazuron (TDZ), a substituted phenyl urea (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea; Dropp TDZ), is a light yellow crystalline compound that is sparingly soluble in water, highly soluble in ethanol and soluble to varying degrees in other organic solvents including acetone, etc (*see* Appendix A.2) for physiochemical properties of TDZ).

Structurally, TDZ is quite different from either auxins or adenine type of cytokinins (*see* Appendix A.1). There are two functional groups in the TDZ molecule, phenyl and thidiazol groups, and the replacement of either of these groups with other ring structures results in the reduction in activity (Mok *et al.*, 1982). Thidiazuron exhibits a high level of activity at concentrations as low as 10 p*M* (Preece *et al.*, 1991) and exposure of plant tissue to TDZ for a relatively short period is sufficient to stimulate regeneration (Visser *et al.*, 1992; Hutchinson and Saxena, 1996a). Thidiazuron has been shown to induce a variety of morphogenic responses with a high degree of efficiency (Fiola *et al.*, 1990; Malik and Saxena, 1992a; Saxena *et al.*, 1992). Thidiazuron has been reported to influence *in vitro* growth responses in plants (Murthy *et al.*, 1998).

Callogenesis

There are limited reports on TDZ mediated callus induction. Capelle *et al.* (1983) reported the induction of callus in mung bean (*Phaseolus lunatus*) in medium supplemented with TDZ and obtained higher proliferation compared to other growth regulators. Likewise, Lin *et al.* (1989) observed callus formation from grape (*Vitis acutifolia*) bud cuttings cultured in medium consisting of TDZ. Murthy and Saxena (1998) showed that at low concentration, TDZ induced green, compact callus in

neem. Callus formation was also observed in cotton leaf disks cultured in medium supplemented with TDZ (Jayashankar *et al.*, 1991). To our knowledge, there are no reports on TDZ-mediated callus induction in the selected ornamental plants (*Polianthes tuberosa* L., *Alstroemeria* cv. Rosita, Carnation cv Yair, and *Ornithogolum* spp.). Nevertheless, Frey and Janick (1991), observed shoot proliferation from organogenic callus induced on calyces and nodal explants in medium fortified with a combination of 0.05µM TDZ and 0.5µM NAA.

Organogenesis

The formation of auxillary buds and adventitious shoots is influenced by growth regulators, especially cytokinins (Muthy *et al.*, 1998). Several authors have reported TDZ induced shoot proliferation in numerous species ranging from root crops to trees (Huetteman and Preece, 1993; Lu, 1993).

A review of previous studies support the hypothesis that TDZ at much lower concentrations, induced shoot regeneration with an efficiency comparable to or greater than that of other cytokinins. Addition of TDZ in culture media resulted in shoot proliferation in bean (*Phaseolus vulgaris*) (Malik and Saxena, 1992a), pea (*Pisum sativum*), chickpea (*Cicer arietinum*), and lentil (*Lens culinaris*) (Malik and Saxena, 1992b), rubus (Fiola *et al.*, 1990), *Acer* x *free-manii* (Kerns and Meyer, 1986). Previous reports suggest that TDZ has been used more effectively in the regeneration of shoots in woody species (Lu, 1993). Thidiazuron also stimulated shoot proliferation in Azaleas (Briggs *et al.*, 1988), silver maple (Preece *et al.*, 1991) and quince (Baker and Bhatia, 1993).

Currently, a few studies have been done on the regeneration of buds and shoots in a number of ornamental plants using TDZ as a growth-regulating substance. In a previous study, TDZ stimulated both callus growth and shoot primodia in *Hibiscus*

and *Syriacus* L. by suspension cultures at 0.1 mg L^{-1} and 0.01 mg L^{-1} respectively (Yoo-Eun Ha *et al* ., 1996).

Multiple shoots from bulb scale explants of *Lilium concolor* var. 'Parthneion' were produced when TDZ at 0.01 mg L^{-1} was added on MS medium (Park *et al.*, 1996). Another ornamental corm, *Liatris* was studied by Stimart and Mather (1996) using TDZ to induce adventitious shoots. The best TDZ concentration was 2.2μM. The shoots subsequently rooted when transferred to MS with 5.0μM IBA. Limited studies have been reported on the induction of shoots in the selected ornamental plants used in the present study.

Watad *et al.* (1996) used TDZ in adventitious shoot regeneration studies from stem explants of carnation cultivar White Sim using 3 different culture procedures: agar-gelled medium, liquid- shaken medium, and an interfacial membrane raft floating on liquid medium. Maximum shoot regeneration was on the raft in the presence of 1.1μM TDZ, with explants derived from the first upper internode. Similarly, Zuker *et al.* (1995), achieved *in- vitro* adventitious shoot organogenesis from stem segments of carnation cv. White Sim using TDZ during transformation by microprojectile bombardment. Frey and Janick (1991), demonstrated shoot proliferation using a combination of 0.05μM TDZ+0.5μM NAA, directly from petal explants and indirectly from calyces and nodal explants of carnation cultivars Scania, improved White Sim and Sandra. However, there are no reports on TDZ-mediated shoot regeneration from carnation cv. 'Yair' shoot tip explants, used in the present study. In addition, there is no information on the effect of dose and duration of exposure to TDZ on the regeneration of shoots of carnation cv. 'Yair' shoot-tip cultures.

A few reports are available on TDZ mediated shoot regeneration in *Alstroemeria*. Hutchinson *et al.* (1994) reported the induction of multiple shoots from embryo-induced callus of a tetraploid *Alstoemeria* (A. Pelegrina x A. Psittacina), using 0.5μM TDZ and 8.0μM BAP in the culture medium. Lin *et al.* (1997) developed a two-step protocol for the induction of shoots from *Alstroemeria* genotype VV024 (a tetraploid breeding line from Van Staaveren BV, The Netherlands) leaf explants. The best induction was obtained with Murashige and Skoog medium containing 10μM TDZ and 0.5μM IBA and regeneration medium containing BAP at 2.2μM. To our knowledge, no reports are available on TDZ-mediated shoot regeneration from *Alstroemeria* hybrid cv. Rosita shoot-tip explants. Furthermore, no studies are available on TDZ-induced regeneration of shoots in *Polianthes tuberosa* and *Ornithogolum* spp. In addition to eliciting organogenic responses in plants, TDZ has been shown to evoke somatic embryogenesis in a number of plant species.

Visser *et al.* (1992) showed for the first time that TDZ substituted for and had a much higher efficiency than that of the auxin-cytokinin complement used in previous procedures to induce somatic embryogenesis in geranium hypocotyl cultures. In addition, they also demonstrated the possible involvement of TDZ in the modulation of endogenous auxins in TDZ-induced somatic embryogenesis. An exposure to TDZ for only 2d, although at a higher concentration (5 μM) than when continuously subjected to TDZ, was sufficient to evoke an embryogenic response in the geranium hypocotyl sections. Simillar results were reported in cultured hypocotyl explants of geranium (*Pelargonium* x *hortorum*) (Hutchinson *et al.*, 1996a, 1996b, 1997a, 1997b; Murthy *et al.*, 1996a). Studies by Hutchinson *et al.* (1997a and 1996b) reported best results in somatic embryogenesis with duration of exposure to 10μM TDZ for 3 d in geranium hypocotyl sections. Hutchinson *et al.* (1996a) obtained the best induction of

somatic embryos continuously cultured in medium supplemented with 0.4μM TDZ. There are no reports on TDZ mediated somatic embryogenesis in the ornamental plants selected for the current study.

Despite the numerous morphogenic responses in which TDZ plays a considerable role, the precise mode of action of TDZ remains to be elucidated.

Thidiazuron and its mode of action

In most of the morphogenic responses mediated by TDZ, it is suggested that TDZ may be acting directly as a cytokinin (Mok and Mok, 1985; Mok *et al.*, 1982; Thomas and Katterman, 1986), and or through the modulation of endogenous plant growth substances: auxins, cytokinins, ethylene, ABA, GAs etc (Hutchinson *et al.*, 1996a, 1996b, 1997a, 1997b).

CHAPTER THREE

The potential of using thidiazuron in *in-vitro* propagation of "Tuberose"(*Polianthes tuberosa* L.)

Abstract:

Whole shoot regeneration was achieved in shoot-tip cultures of Tuberose (*Polianthes tuberosa* L.) inoculated on media supplemented with various concentrations of thidiazuron (TDZ). TDZ at 0.1 to 1μM induced shoot elongation with efficiency greater than that obtained by conventionally used combination of BAP and NAA. However, the results were comparable to those obtained in medium supplemented with BAP alone. Addition of NAA to TDZ had similar results to a combination of BAP and NAA by inducing callus formation.

Introduction

Tuberose (*Polianthes tuberosa* L.) was introduced in Europe from Mexico, which is the center of origin. True blood, (1973), Rose, (1903-1905) described 12 wild species of tuberose in Mexico and nine had white flowers. Tuberose has become a popular cut-flower in Kenya, especially with small to medium scale farmers, due to it's simple management practices and ease of growth outdoors (Chebet, 1999). It is thought to have been introduced in Kenya in the late 1960's to early 1970's by the white settlers (HCDA, 1994). In Kenya, the known varieties include; the Pearl, Dwarf pearl, Mexico early, Ever blooming, Pleno and Single or Mexican single, with the Pearl being more popular among farmers.

Tuberose grows well in upper highlands, where soil temperature is usually between 10 to 15°C). The crop requires warm temperatures for growth of flowers (25°C). It does well under irrigation since it is a poor competitor for water. In Kenya, tuberose is grown around Limuru in Kiambu district. Other suitable areas include Kinangop, Eldoret, Bungoma and Kitale.

Polianthes belongs to the subclass monocotyledonae. Baker (1888) classified polianthes in the family of Amaryllidaceae, suborder III, the Agavae. Hutchinson (1934) placed the genus in the family Agavaceae. Cytological studies supported this classification (Joshi and Pantulu, 1941; Sato, 1942; Sharma and Gosh, 1956).

Tuberose is a herbaceous perennial, which produces fragrant flowers, with a creamy white colour. It can grow up to 3 years under good management. The shoots arise from an underground tunicate bulb. The roots are fibrous or thick and fleshy. The leaves have parallel veins, are green in colour with a purplish tinge.

The inflorescence is a spike (Chebet, 1999). Each spike can consist of several flowers of up to twelve (Bailey, 1961; Benschop, 1993). There are six stamens on the perianth, the anthers are dorsifixed in the middle, the ovary locular, and the ovules numerous (Hutchinson, 1934). Tuberose is described as a tuberous rhizome by Rose (1903-1905). Bryan, (1989) referred to them as tubcrosc bulbs.

Tuberose produces seeds that are difficult to germinate, as a result, it is propagated by bulbs. The weight of the bulb should be 30g and above, while optimum storage conditions of (30-35°C) promote good flowering, sprouting and leaf growth. Planting should be done during the rainy season (April/May) in Kenya. However, if the crop is grown under irrigation, then the planting season should be such that markets are targeted well because the crop can be grown during any season (HCDA, 1994).

Tuberose (*Polianthe tuberosa* L.) has become increasingly important as a cut-flower (Chebet, 1999; Heywood, 1982; Sanyal *et al.*, 1998; Watako, 1992). Some of the problems reported to affect tuberose include; production of sterile seed, narrow genetic base, slow to propagate vegetatively and suffers from fungus and nematode attacks (Yadav and Maity, 1989). Recently, tuberose plants in New Zealand have been reported to exhibit a leaf mottling symptom resembling a virus (Benschop, 1993).

In vitro techniques have been used for rapid clonal multiplication of superior clones, production of clean materials, *in vitro* conservation and cryopreservation, international exchange of germplasm and biotechnological applications (Hartman, 1985). In addition, the full potential of micropropagation can only be realised if efficient protocols are developed (Dodds, 1989; Kitto, 1997). This can be achieved through manipulation of the culture environment, nutrient salts in the medium, genotype, type of explant, regeneration pathways and plant growth regulators.

Conventionally, the manipulation of PGRs has proved to be faster, cheaper, simpler method of achieving efficient protocols. Traditionally, a combination of auxins and cytokinins based on Skoog and Miller (1957) hypothesis, has been used in most regeneration systems. In recent years, an *in vitro* system has been developed for multiplication of tuberose using a combination of auxin and cytokinin. Bulb segments are used and up to 800 plants can be regenerated from a single bulb. Growing conditions included temperatures of 25±2°C using continuous florescent light of approximately 3500 lux and a combination of BAP+NAA (Benschop, 1993). Similarly, Tuberose has been tissue cultured using MS salts, NAA and BA (Sanyal *et al.*, 1998;Waithaka, 1986). These researchers reported on the regeneration of shoots from leaf induced callus cultures. Sanyal *et al.* (1998) observed shoot regeneration

and the formation of buds from callus tissue in medium containing NAA (0.2-0.5)mg L^{-1} in combination with 2.0mg L^{-1} BA. There are no reports on the *in vitro* culture of tuberose shoot-tip explants to provide clean planting materials, facilitate the conservation and exchange of germplasm and genetic manipulation (Rabinowich and Brewster, 1990).

Thidiazuron, a substituted phenyl urea (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea; Dropp TDZ), which was primarily used as a cotton (*Gossypium hirsutum* L.) defoliant (Bruce *et al.*, 1965; Arndt *et al.*, 1976), has been shown in various regeneration systems to evoke a comparable or higher degree of growth response than adenine-based cytokinins or a combination of auxin and cytokinin (Capelle *et al.*, 1983; Thomas and Katterman, 1986; Mok *et al.*, 1982). Besides, TDZ has been effectively used to induce shoot regeneration on leaf explants of many dicots (Huetteman and Preece 1993; Turk *et al.*, 1994; Dubois and de Vries 1995), but it's use has not been reported fully in monocots, including tuberose.

The main objective of this section of the study was to determine the role of TDZ in morphogenesis and plant regeneration in tuberose shoot-tip cultures.

The specific objectives were:

(i) to investigate the effect of TDZ compared to a combination of BAP and NAA on morphogenesis and regeneration of plantlets from tuberose shoot-tip cultures.

(ii) to assess the influence of auxins on rooting of tuberose shoots

Materials and Methods

Site

Tuberose stock plants with both shoots and bulbs were obtained from Mbugwa farm in Limuru. Limuru is at an altitude of 1800-2100m. a.s.l. The area experiences a bimodal distribution of rainfall, with long rains in March-May and short rains in October-December. The annual rainfall is between 700-1000mm. The mean maximum day temperature is about 23°C and mean minimum of 15°C.

Plant Material

Tuberose was grown under outdoor conditions with minimal supplemental irrigation during the dry seasons (January-March) and (July-September). Bulbs were dipped in benlate before planting at 5-10cm deep, on 1m wide raised beds. During land preparation, organic manure from poultry droppings and wood shavings was incorporated with DAP at 125kg/ha. The bulbs were planted at a spacing of 15-20cm between rows and 30cm within rows depending on the soil status, making 4 rows per 1m wide bed. Manual weeding was done to keep the beds weed free. The crop was sprayed against fungal infections (stem rot, botrytis e.t.c.).and spider mites using Dithane M45 and Rogor E, respectively. Top dressing with CAN was done at 100 kg N/ha in a split application at 30 and 60 days after planting.

Preparation of explant and sterilisation

Tuberose bulbs were obtained from three months old crop. The bulbs were cleaned with detergent and rinsed in running tap water for 15 minutes. Excised tips (1-2cm) long were placed in tap water in a beaker. The water was drained and the tips were immersed for 5 minutes in 95% alcohol. The alcohol was drained and the tips were rinsed in sterile distilled water for 3 minutes. The tips were placed in 0.5% NaOCl containing 'Tween 20' (2 drops/ 100mL solution), for 20 minutes, washed in

three changes of distilled water and placed in a dry sterile petri dish before, shoot-tips (0.5-1.0mm long), consisting of an apical dome and one to two leaf primodia were excised under a dissecting microscope and explanted.

Culture of shoot-tip explants

Each explant was cultured per universal bottle containing 10mL of medium. The medium consisted of MS (Murashige and Skoog, 1962) salts, B5 (Gamborg et al., 1968) vitamins, 30 g/l sucrose and PGRs.

A total of 16 treatments, each replicated four times and arranged in a completely randomised design (CRD) were used as follows:

1. MSO
2. 0.1μM TDZ
3. 0.4μM TDZ
4. 1.0μM TDZ
5. 5.0μM TDZ
6. 0.05mg L^{-1} BAP
7. 0.1mg L^{-1} BAP
8. 1.0mg L^{-1} BAP
9. 0.1mg L^{-1} BAP+0.01mg L^{-1} NAA
10. 1mg L^{-1} BAP+0.01mg L^{-1} NAA
11. 1mg L^{-1} BAP+0.1mg L^{-1} NAA
12. 0.1 μM TDZ+0.01mg L^{-1} NAA
13. 1 μM TDZ+0.01mg L^{-1} NAA
14. 1 μM TDZ+0.1mg L^{-1} NAA
15. 0.01mg L^{-1}NAA
16. 0.1mg L^{-1}NAA

The explants were maintained on the medium for four weeks after which they were sub-cultured after every month to the same medium except shoots previously cultured in medium containing TDZ alone or in combination with NAA. Shoots from the later media, were exposed to TDZ treatments for 10 days before they were transferred to MSO medium. At the end of the twelfth week, the shoots were

transferred to rooting medium with or without auxins. Sub-culturing in rooting medium was done after every four weeks. The pH of all media was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 20 min. The cultures were placed on shelves set at 25± 2°C and illuminated (16-hour photoperiod; 70-78umol $m^{-2}s^{-1}$) by cool-white fluorescent tubes. The treatments for rooting medium consisted of 0.1mg L^{-1} NAA, 1.0mg L^{-1}NAA, 0.1mg L^{-1} IBA, and MSO. In the last experiment which investigated the effect of the previous medium on subsequent rooting, nine shoots, with a single shoot in each universal bottle and replicated three times per treatment were used.

Shoot length, number of leaves and shoot quality rating were assessed weekly for three months. Shoot quality rating was scored (using a scale 0-4), 0-poor growth, poor chlorophyll accumulation and poor colour (browning of tissues), 4-the best growth, chlorophyll accumulation and colour (green). Scores on rooting were based on the following: (-) =no rooting, + =low rooting (<3 roots per surviving shoot), ++ =fair rooting (3-5 roots per surviving shoot), +++ =good rooting (5-10 roots per surviving shoot), ++++ =very good rooting (>10 roots per surviving shoot).

Statistical analysis

In the first experiment, on the effect of TDZ alone at various concentrations or in combination with NAA on morphogenic responses compared to a combination of BAP and NAA consisted of four replicates per treatment. The second experiment on the influence of auxins on rooting of tuberose shoots had 3 replicates per treatment combination. Data on percent rooting of plantlets were subjected to arc sine ($\sqrt{x}/100$) prior to statistical analyses. The experiments were repeated at least twice. Data were analysed using analysis of variance (GENSTAT statistical software (Lane and Payne, 1996), and the means were compared by Turkey's procedure at 5% level of probability.

Results

Shoot length

The PGRs had a highly significant effect on the mean shoot length (Table 3.1). Multiple shoots were not observed in any of the treatments. Culture medium supplemented with NAA at 0.01 and 0.1mg L^{-1} alone was not included in the results, because the shoot-tip explants turned brown and died after 5 days in culture. There was a general increase in shoot elongation in most of the treatments, except medium containing a combination of TDZ+NAA and BAP+NAA, where a decline in shoot elongation was observed from 3 to 8 weeks in culture (Fig. 3.1; Table 3.1). At 8 to 12 weeks in culture, all the shoot tissues in medium containing a combination of TDZ+NAA and BAP+NAA changed into friable to compact cream to green callus.

Basal MSO medium supported growth of shoots for the 12 weeks culture period. However, there was a slight increase in shoot length, from 0.125 to 1.075cm, during the 12 weeks in culture compared to the other treatments (Fig. 3.1, Table 3.1). A low TDZ concentration of 0.1μM had no significant increase on shoot elongation from 1 to 5 weeks in culture (Fig. 3.1, Table 3.1). However, from 8 to 12 weeks in culture, a significant increase on shoot length 1.275 to 3.05cm respectively, was observed (Fig. 3.1, Table 3.1).

Medium containing 1.0μM TDZ significantly increased the shoot length, and had the highest shoot lengths (0.45, 1.125, 1.475, 2.3, 3.175, and 5.15) cm, at (1, 3, 5, 8, 10, and 12) weeks in culture respectively (Fig. 3.1, Table. 3.1, Plate 1). These results were not significantly different from those recorded in medium containing 0.4μM TDZ at 3, 5, and 12 weeks in culture (Table 3.1). Higher levels of TDZ (5.0μM) had no significant effect on the increase on shoot elongation in the first 8 weeks of culture (Fig. 3.1 Table 3.1).

Table. 3.1 Effect of TDZ alone at various concentrations or in combination with NAA on the mean shoot length (cm) compared to a combination of BAP and NAA in (*Polianthes tuberosa* L.) shoot-tip cultures.

	weeks in culture					
PGRs	1	3	5	8	10	12
MSO	0.125c	0.5bcd	0.525def	0.775de	0.825d	1.075c
0.1μM TDZ	0.175c	0.625bc	0.825bcd	1.275c	1.775bc	3.05b
0.4μM TDZ	0.35b	0.85ab	1.15ab	1.85b	2.325b	4.55a
1.0μM TDZ	0.45a	1.125a	1.475a	2.3a	3.175a	5.15a
5.0μM TDZ	0.125c	0.55bcd	0.6de	1.1cd	1.8bc	2.55b
0.05mg/l BAP	0.15c	0.65bc	1.025bc	1.675b	2.2b	2.925b
0.1mg/l BAP	0.175c	0.45bcd	0.75cd	1.05cd	1.375cd	1.575c
1.0mg/l BAP	0.125c	0.35cd	0.45df	0.6e	0.975d	1.375c
0.1mg L^{-1} BAP+0.01mg L^{-1} NAA	0.175c	0.25cd	0.05g	0f [x]	0e [x]	0d [x]
1mg L^{-1} BAP+0.01mg L^{-1} NAA	0.2c	0.6bcd	0.3efg	0f	0e	0d
1mg L^{-1} BAP+0.1mg L^{-1} NAA	0.125c	0.35cd	0.25efg	0f	0e	0d
0.1 μM TDZ+0.01mg L^{-1} NAA	0.175c	0.175d	0.05g	0f	0e	0d
1 μM TDZ+0.01mg L^{-1} NAA	0.15c	0.3cd	0.25efg	0f	0e	0d
1 μM TDZ+0.1mg L^{-1} NAA	0.2c	0.325cd	0.2fg	0f	0e	0d
SE	0.04	0.17	0.16	0.13	0.22	0.25
W (Turkey's)	0.1	0.44	0.4	0.34	0.56	0.62
Significance	***	***	***	***	***	***

*** =Significant at $P \leq 0.001$.
Y = Mean separation using Turkey's. Values having the same letters within the same column are not significantly different at 5% level of probability
[x]= Entire shoot tissues changed to callus

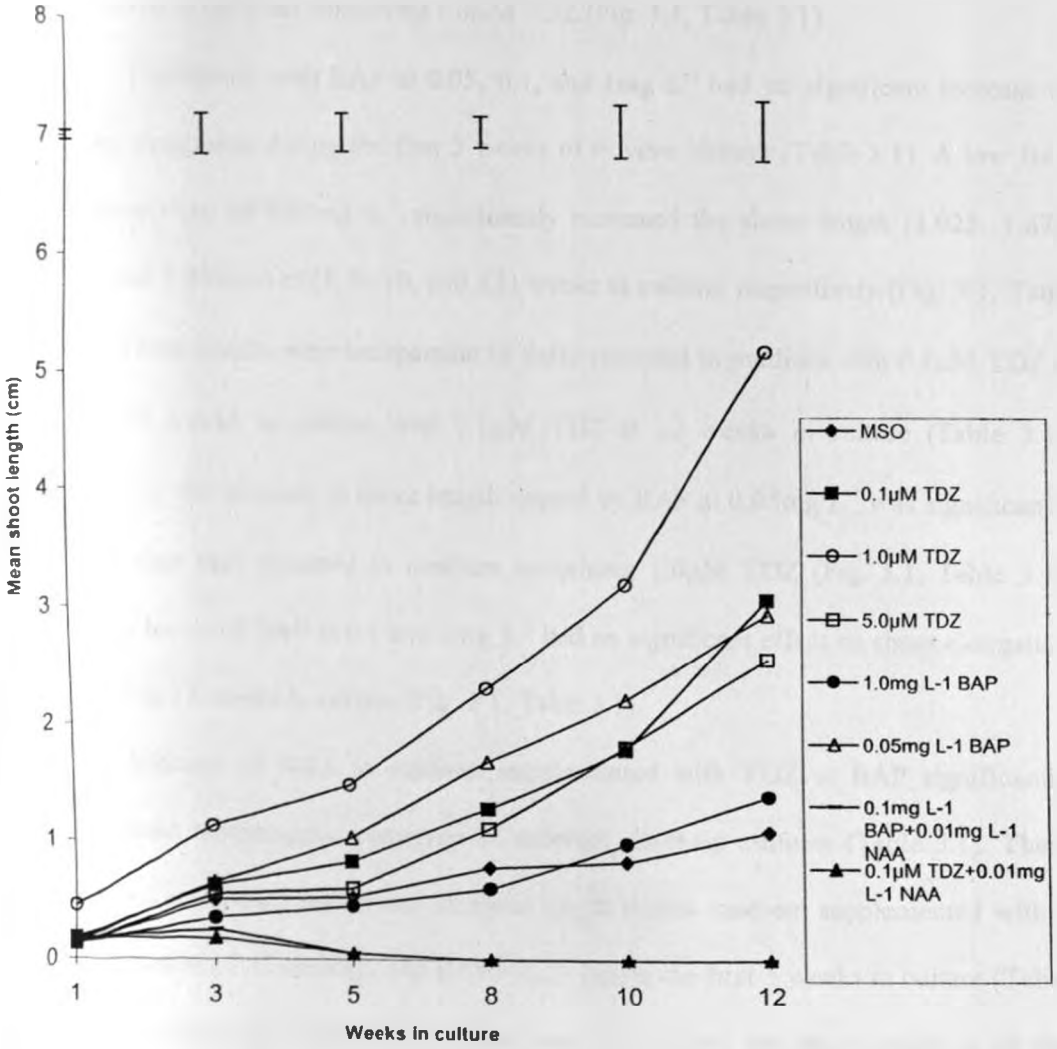


Fig. 3.1 Effect of TDZ alone at various concentrations or in combination with NAA on the mean shoot length (cm) compared to a combination of BAP and NAA in *Polianthes tuberosa* L. shoot-tip cultures. Vertical bars represent standard error

However, a significant increase in the shoot lengths of (1.8 and 2.55cm) over the control, was noticed at 10 and 12 weeks in culture respectively, and was comparable to that observed in medium containing 0.1μM TDZ during the same culture period (Table 3.1). Furthermore, these results were significantly low in relationship to that observed in medium containing 1.0μM TDZ (Fig. 3.1, Table 3.1).

Treatments with BAP at 0.05, 0.1, and 1mg L^{-1} had no significant increase on shoot elongation during the first 3 weeks of *in vitro* culture (Table 3.1). A low BAP concentration of 0.05mg L^{-1} significantly increased the shoot length (1.025, 1.675, 2.2, and 2.925cm) at (5, 8, 10, and 12) weeks in culture, respectively (Fig. 3.1, Table 3.1). These results were comparable to those recorded in medium with 0.4μM TDZ at 5 to 10 weeks in culture, and 0.1μM TDZ at 12 weeks in culture (Table 3.1). However, the increase in shoot length caused by BAP at 0.05mg L^{-1} was significantly lower than that observed in medium containing 1.0μM TDZ (Fig. 3.1, Table 3.1). Higher levels of BAP at 0.1 and 1mg L^{-1} had no significant effect on shoot elongation from 5 to 12 weeks in culture (Fig. 3.1, Table 3.1).

Addition of NAA to medium supplemented with TDZ or BAP significantly influenced morphogenic responses in tuberose shoot-tip cultures (Table 3.1). There were no significant differences in shoot length within medium supplemented with a combination of TDZ+NAA and BAP+NAA during the first 5 weeks in culture (Table 3.1). However, no significant increase was observed on the shoot length in all the treatments in the first three weeks in culture (Table 3.1). Furthermore, a significant decrease in shoot length (0.05cm) was noticed in medium containing 0.1mg L^{-1} BAP+0.01mg L^{-1} NAA and 0.1μM TDZ +0.01mg L^{-1} NAA at 5 weeks in culture, and in all the media containing a combination of TDZ+NAA and BAP+NAA from 8 to 12 weeks in culture (Fig. 3.1, Table 3.1). From 8-12 weeks, no increase in shoot length

was reported in medium containing a combination of TDZ+NAA and BAP+NAA (Table 3.1).

TDZ at 1.0μM significantly increased shoot elongation compared to BAP at 0.05, 0.1 and 1mg L^{-1}. A combination of BAP and NAA had a significant effect on morphogenic responses in tuberose shoot tip cultures, through induction of callus that was friable to compact in texture, and cream to green callus in colour. In addition, a combination of BAP and NAA inhibited shoot elongation.

Shoot quality rating

Basal medium supported poor quality of shoots 2.75-1.75 (pale-light green) shoots during the last four weeks in culture. The PGRs had a significant effect on the quality of shoots (Table 3.2). In the first week of culture, the PGRs had no significant effect on the quality of shoots, and generally low shoot quality rating was observed (Table 3.2). Moderate (3) to good (4) shoot quality rating was recorded in most of the media except medium supplemented with MSO, BAP+NAA and TDZ+NAA throughout the culture period (Fig. 3.2, Table 3.2). MSO medium supported significantly high shoot quality rating (3) in the 3rd and 5th week in culture (Table 3.2). However, in subsequent weeks, shoot quality rating declined up to 1.75 at 12 weeks in culture (Fig. 3.2, Table 3.2).

Thidiazuron at (0.1-5.0)μM had no significant increase on the shoot quality rating in the first 5 weeks in culture (Fig. 3.2, Table 3.2). However, at 8 to 12 weeks

Table. 3.2 Effect of TDZ alone at various concentrations or in combination with NAA on the mean shoot quality rating[z] compared to a combination of BAP and NAA in (*Polianthes tuberosa* L.) Shoot-tip cultures.

PGRs	weeks in culture					
	1	3	5	8	10	12
MSO	2.25	3a	3abc	2.75c	1.75c	1.75c
0.1μM TDZ	2.5	3.75a	3.75ab	3.5abc	3.5ab	3.25b
0.4μM TDZ	2	3.5a	3.5ab	3.25abc	3.25ab	3b
1.0μM TDZ	2.75	4a	4a	3.5abc	3.5ab	3.25b
5.0μM TDZ	2.75	4a	4a	4a	4a	4a
0.05mg/l BAP	3	3.25a	3.25abc	3bc	3b	3b
0.1mg/l BAP	2.25	3.75a	3.75ab	3.75ab	3.75ab	3.5ab
1.0mg/l BAP	2.5	3.5a	3.5ab	4a	4a	4a
0.1mg L^{-1} BAP+0.01mg L^{-1} NAA	2.75	2ab	1d	0d	0d	0d
1mg L^{-1} BAP+0.01mg L^{-1} NAA	2.75	2.75ab	2.25abcd	0d	0d	0d
1mg L^{-1} BAP+0.1mg L^{-1} NAA	3	2.25ab	2bcd	0d	0d	0d
0.1 μM TDZ+0.01mg L^{-1} NAA	2.25	1b	1d	0d	0d	0d
1 μM TDZ+0.01mg L^{-1} NAA	2.75	2.25ab	2bcd	0d	0d	0d
1 μM TDZ+0.1mg L^{-1} NAA	2.75	2.25ab	1.5cd	0d	0d	0d
SE	0.45	0.85	0.8	0.31	0.3	0.27
W (Turkey's)	-	2.25	2	0.8	0.8	0.66
Significance	n.s	***	***	***	***	***

[z] Visual estimates based on colour, and appearance on a scale of 0 to 4 where 0=no shoot, 1= brown, 2=pale green, 3-light green and 4-green good appearance

n s,*** =nonsignificant , significant at $P \leq 0.001$ respectively

Y = Mean separation using Turkey's. Values having the same letters within the same column are not significantly different at 5% level of probability

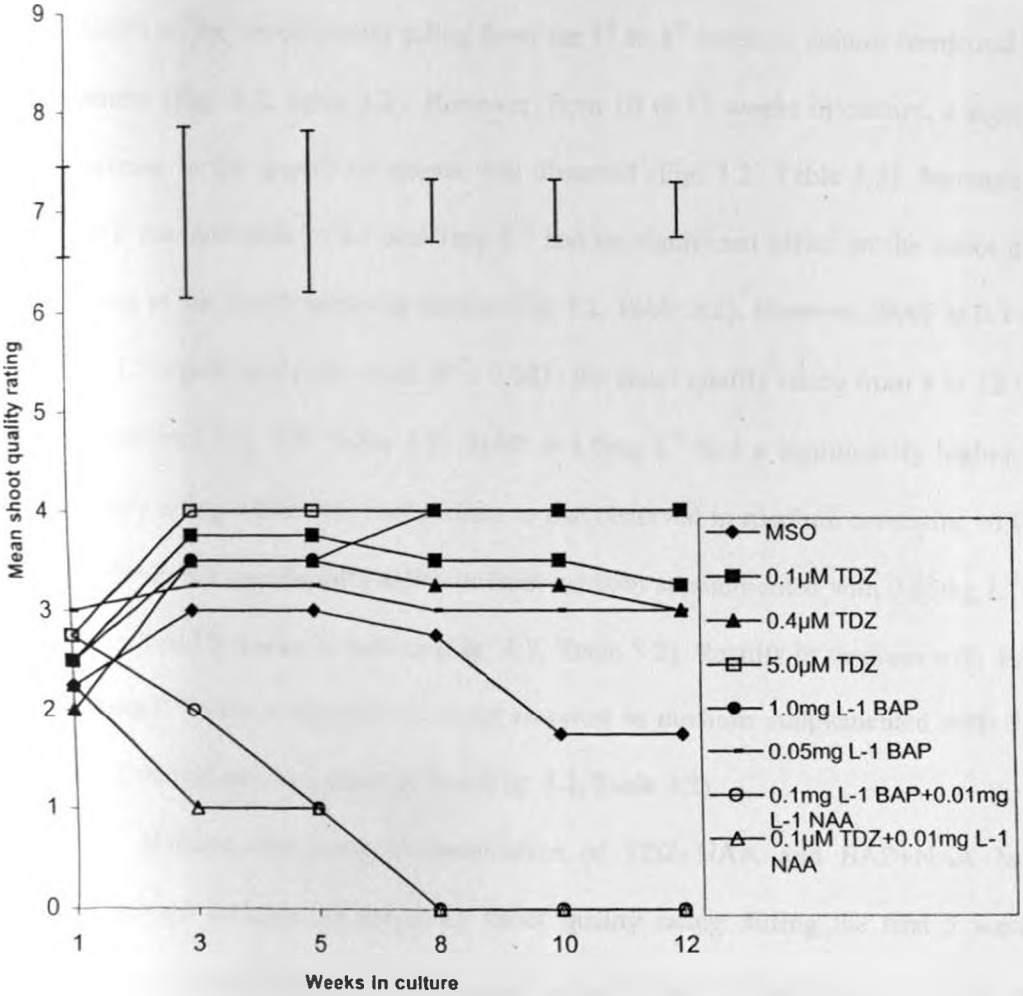


Fig 3.2 Effect of TDZ alone at various concentrations or in combination with NAA on the mean shoot quality rating compared to a combination of BAP and NAA in *Polianthes tuberosa* shoot-tip cultures. Vertical bars represent standard error

in culture, TDZ at various concentrations significantly increased the shoot quality rating (Fig. 3.2, Table 3.2). TDZ at 5.0μM supported a significantly higher shoot quality rating (4) compared to medium containing (0.1, 0.4, and 1.0) μM TDZ at 12 weeks in culture (Fig. 3.2, Table 3.2).

N^6 -Benzylaminopurine (BAP) at 0.05mg L^{-1}, had no significant increase ($P \leq 0.001$) on the shoot quality rating from the 1st to 8th week in culture compared to the control (Fig. 3.2, Table 3.2). However, from 10 to 12 weeks in culture, a significant increase in the quality of shoots was observed (Fig. 3.2, Table 3.2). Increasing the BAP concentration to 0.1 and 1mg L^{-1} had no significant effect on the shoot quality rating in the first 5 weeks in culture (Fig 3.2, Table 3.2). However, BAP at 0.1 and 1 mg L^{-1} significantly increased ($P \leq 0.001$) the shoot quality rating from 8 to 12 weeks in culture (Fig. 3.2, Table 3.2). BAP at 1.0mg L^{-1} had a significantly higher shoot quality rating which was comparable to that observed in medium consisting of 0.1mg L^{-1} BAP, but significantly different from medium supplemented with 0.05mg L^{-1} BAP from 8 to 12 weeks in culture (Fig. 3.2, Table 3.2). Results in medium with BAP at 1.0mg L^{-1} were comparable to those recorded in medium supplemented with 5.0μM TDZ throughout the culture period (Fig. 3.2, Table 3.2).

Medium containing a combination of TDZ+NAA and BAP+NAA had no significant increase on the mean shoot quality rating during the first 5 weeks in culture, except medium supplemented with 0.1μM TDZ+0.01mg L^{-1} NAA which significantly lowered the mean shoot quality rating (1), resulting in browning of tissues. in the 3rd and 5th week in culture (Fig. 3.2, Table 3.2). A combination of TDZ+NAA and BAP+NAA inhibited shoot elongation and instead induced formation of callus from entire shoot tissues from 8 to 12 weeks in culture (Table 3.2). Data with zero rating indicate no shoot (Table 3.2).

TDZ at 5.0μM significantly increased the quality of shoots, and the results were comparable to those observed in medium consisting of BAP at higher levels (0.1 to 1.0mg L^{-1}). A combination of BAP and NAA in the medium significantly lowered the quality of shoots.

Number of leaves

Highly significant differences in the number of leaves were detected by analysis of variance among the PGRs, except for the first week in culture (Table. 3.3). An increase in the number of leaves was observed in all treatments except medium containing a combination of TDZ+NAA and BAP+NAA, where a decline in the number of leaves was noticed during the culture period (Fig. 3.3). Medium without any PGRs supported very few healthy, well developed leaves throughout the culture period (Fig. 3.3, Table 3.3).

Low TDZ concentrations at 0.1, 0.4, and 1.0μM significantly increased ($P \leq 0.001$) the number of leaves compared to the control, from 3 to 12 weeks in culture (Fig. 3.3, Table 3.3). TDZ at 1.0μM produced the highest number of leaves (5.25) compared to TDZ at 0.1 and 0.4μM in the 12th week in culture (Fig. 3.3, Table 3.3, Plate 1). A high TDZ concentration of 5.0μM caused no significant increase in the number of leaves relative to MSO medium, from the 3rd to the 10th week in culture (Fig. 3.3, Table 3.3). Increase in the number of leaves (3) relative to the control, was observed at 12 weeks in culture, and was similar to that recorded in medium containing 0.1 and 0.4μM TDZ (Table 3.3).

Table. 3.3 Effect of TDZ alone at various concentrations or in combination with NAA on the mean number of leaves per shoot compared to a combination of BAP and NAA in (*Polianthes tuberosa* L.) shoot-tip cultures.

PGRs	weeks in culture					
	1	3	5	8	10	12
MSO	1	1cd	1.25cdef	1.75b	1.5c	1.5d
0.1μM TDZ	1	2.75a	2.75ab	3.25a	3.25ab	3.75b
0.4μM TDZ	1	2.5ab	2.5abc	3.25a	3.25ab	3.25bc
1.0μM TDZ	1	2.75a	3.5a	3.5a	3.75a	5.25a
5.0μM TDZ	1	1.75abc	1.75bcde	2.5ab	2.5bc	3bc
0.05mg/l BAP	1	1.75abc	2.25abcd	2.75ab	3ab	3.25bc
0.1mg/l BAP	1	1.5bcd	2.25abcd	2.25b	2.5bc	2.5cd
1.0mg/l BAP	1	1.25cd	1.5bcdef	1.5b	2.5bc	3bc
0.1mg L^{-1} BAP+0.01mg L^{-1} NAA	1	0.75cd	0.25f	0c[x]	0d[x]	0e[x]
1mg L^{-1} BAP+0.01mg L^{-1} NAA	1	1cd	1def	0c	0d	0e
1mg L^{-1} BAP+0.1mg L^{-1} NAA	1	0.75cd	0.75ef	0c	0d	0e
0.1 μM TDZ+0.01mg L^{-1} NAA	1	0.5d	0.5ef	0c	0d	0e
1 μM TDZ+0.01mg L^{-1} NAA	1	0.75cd	0.75ef	0c	0d	0e
1 μM TDZ+0.1mg L^{-1} NAA	1	0.75cd	0.75ef	0c	0d	0e
SE	0.0	0.4	0.5	0.4	0.44	0.4
W (Turkey's)	-	1.1	1.26	1	1.1	1
Significance	n.s	***	***	***	***	***

n.s,*** =nonsignificant , significant at $P \leq 0.001$ respectively
Y = Mean separation using Turkey's. Values having the same letters within the same column are not significantly different at 5% level of probability
[x]= Entire shoot tissues converted to callus

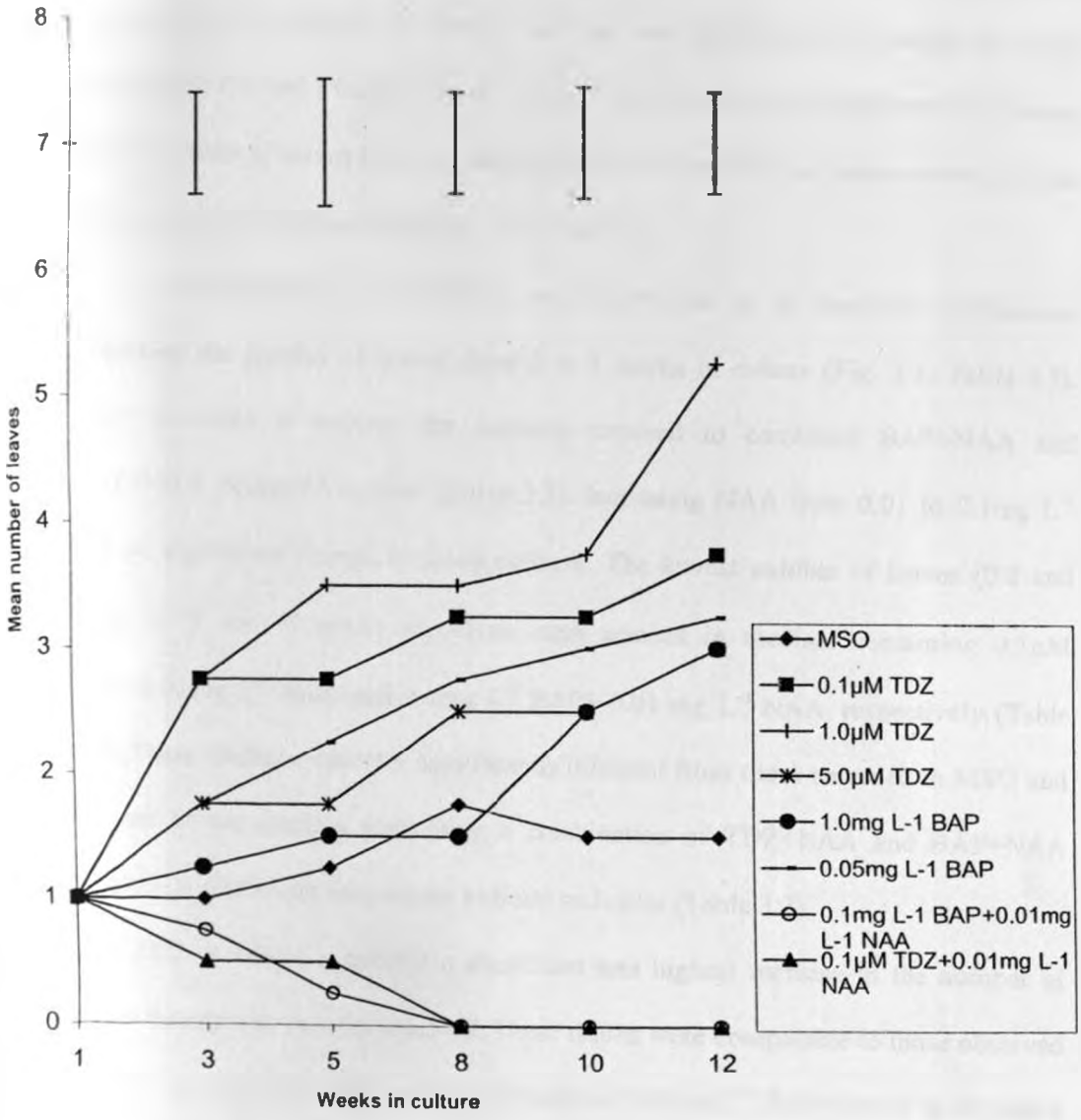


Fig. 3.3 Effect of TDZ alone at various concentrations or in combination with NAA on the mean number of leaves compared to a combination of BAP and NAA in *Polianthes tuberosa* L. shoot-tip cultures. Vertical bars represent standard error

Medium containing BAP at 0.05, 0.1, and 1mg L^{-1} had no significant increase on the number of leaves over the control, from 3 to 8 weeks in culture (Table 3.3). However, at 10 to 12 weeks in culture, 0.05mg L^{-1} BAP recorded a significant increase in the number of leaves, but was not significantly different from that recorded in 0.1 and 1.0mg L^{-1} BAP (Table 3.3). There were no significant differences in the number of leaves between treatments containing BAP at various concentrations throughout the culture period (Fig. 3.3, Table 3.3).

A combination of TDZ+NAA and BAP+NAA in the medium significantly decreased the number of leaves from 3 to 5 weeks in culture (Fig. 3.3, Table 3.3). After 8 weeks in culture, the explants exposed to combined BAP+NAA and TDZ+NAA changed to callus (Table 3.3). Increasing NAA from 0.01 to 0.1mg L^{-1} had no significant change in these cultures. The lowest number of leaves (0.5 and 0.25) at (3 and 5) weeks in culture were noticed in medium containing 0.1μM TDZ+0.01mg L^{-1} NAA and 0.1mg L^{-1} BAP+ 0.01 mg L^{-1} NAA, respectively (Table 3.3). These findings were not significantly different from those recorded in MSO and the rest of the medium containing a combination of TDZ+NAA and BAP+NAA (Table 3.3). Data with zero values indicate no leaves (Table.3.3).

TDZ at 1.0μM supported a significant and highest increase in the number of leaves throughout the culture period. These results were comparable to those observed in medium containing TDZ at 0.1 to 0.4μM and 0.05mgL^{-1} BAP from 3 to 10 weeks in culture. A combination of BAP and NAA significantly influenced morphogenic responses through induction of friable to compact cream to green callus. In addition, a combination of BAP and NAA significantly lowered the number of leaves.

Rooting

Results from the rooting experiment showed that tuberose rooted in all the media used in the experiment (Table. 3.4).). Basal medium recorded rooting at a higher level (30%) compared to 1.0mg L^{-1} NAA (20%), although the difference was not significant (Table 3.4). The highest rooting (80%) was observed in medium supplemented with 0.1mg L^{-1} IBA and was similar to that observed in medium containing 0.1mg L^{-1} NAA (60%) (Table 3.4). MSO medium supported the growth of some roots (0.65) (Table 3.4). Medium supplemented with 0.1mg L^{-1} NAA recorded a significant, and highest increase in the number of roots (6.32), which was significantly different from that observed in medium containing 0.1mg L^{-1} IBA (2.5) (Table 3.4). Increasing the concentration of NAA to 1.0mg L^{-1} did not significantly increase the number of roots (0.53) in relation to the control (Table 3.4). In addition, results recorded in medium containing 0.1mg L^{-1} IBA were not significantly different from those observed in MSO medium (Table 3.4).

Basal medium recorded a considerable increase in root length (3.4cm) (Table 3.4). Medium containing 1.0mg L^{-1} NAA, 0.1mg L^{-1} NAA, and 0.1mg L^{-1} IBA had no significant increase on the shoot length compared to MSO medium (Table 3.4). Medium amended with NAA at 0.1 and 1.0mg L^{-1}, significantly reduced the root length (Table 3.4).

Table. 3.4 Influence of auxins on mean number of roots and mean root length in regenerated shoots[z] of *Polianthes tuberosa* L.

Medium	Percentage of shoots rooting[z]	Mean number of roots	Mean root length (cm)
MSO*	30.4b[y]	0.65b	3.4a
0.1mg/lIBA	86a	2.5b	2.97a
0.1mg/lNAA	60.6ab	6.32a	0.69b
1.0mg/lNAA	20.1b	0.53b	0.61b
SE	0.25	1.12	0.21
W (Turkey's)	0.499	2.18	0.449

[z] = Actual data are presented, but data were transformed based on arc sin ($\sqrt{}$ x/ 100) values for analysis.
* MSO Medium without any PGRs.
[y] = Mean separation using Turkey's procedure: Values having the same letters within the same column are not significantly different at 5% level of probability

Bulblet formation

The formation of bublets (data not shown) was observed in medium containing 0.4-1.0 μM TDZ (Plate. 2).

Effect of type and level of concentration of PGRs in the media on subsequent rooting of Tuberose shoots

The type and level of concentration of PGRs in the media had an effect on subsequent rooting of tuberose shoots (Table 3.5). Generally, rooting occurred in all shoots except those previously grown in media containing BAP at 0.1mg L^{-1} and 1.0mg L^{-1} and subsequently transferred to medium containing 0.1mg L^{-1} NAA and MSO (Table 3.5). The best rooting was observed in medium with 1.0μM TDZ and shoots subsequently transferred to medium with 0.1mg L^{-1} NAA (Table 3.5, Plate 3). There was rooting in media with 0.1 mg L^{-1} IBA in shoots previously grown in media containing BAP, a case which was not noticed in the other rooting media. Shoots with numerous roots that were also not very long acclimatized better.

Table. 3.5 Effect of type and level of concentration of PGRs in the media on tuberose shoots[z] in subsequent rooting medium.

Previous media	Rooting media		
	0.1 IBA	0.1 NAA	MSO*
MSO	++	+++	+
0.1μM TDZ	+	+	+
0.4μM TDZ	++	++	+
1.0μM TDZ	++	++++	+
5.0μM TDZ	+	++	+
0.05mg L^{-1} BAP	++	–	–
0.1mg L^{-1} BAP	+	–	–
1.0mg L^{-1} BAP	+	–	–

[z] = Shoots previously cultured in medium supplemented with PGRs

(-) =no rooting

+ Low rooting = <3 roots per surviving shoots

++ fair rooting =3-5 roots per surviving shoots

+++ Good rooting = 5-10 roots per surviving shoots

++++ Very good rooting = >10 roots per surviving shoots

Plate 1. *In vitro* regeneration of plants from shoot-tip cultures of *Polianthus tuberosa* L. Shoots cultured in MS medium supplemented with 1.0μM TDZ for 10 days and transferred to MSO medium before subculturing in rooting medium.

Plate 2. *In vitro* regeneration of plants from shoot-tip cultures of *Polianthus tuberosa* L., in MS medium supplemented with 0.4 and 1.0μM TDZ for 10 d and transferred to MSO medium, after 8 weeks in culture.

Plate 3. Rooted tuberose shoots at four weeks, after transfer to rooting medium and previously cultured in TDZ at 1.0μM; left in medium containing IBA at 0.1mg L^{-1} and right in medium containing NAA at 0.1mg L^{-1}.

Plate 4. Establishment of *in vitro* regenerated plants of *Polianthes tuberosa* L., in medium supplemented with 1.0μM TDZ at 2 weeks from initial transfer to sterile soil.

Discussions

High shoot elongation resulted in more number of leaves, conversely, inhibition of shoot elongation resulted in fewer number of leaves. Furthermore, these two variables had no well-defined relationship with the quality of shoots. Perhaps as a result of an inverse relationship observed between relatively high concentrations of PGRs used and shoot elongation.

Shoot length

TDZ or BAP alone in the culture medium supported the survival of shoot-tip explants and caused an increase in shoot elongation during the 12 weeks culture period. In contrast, a combination of TDZ+NAA or BAP+NAA significantly influenced morphogenic responses and induced friable to compact, cream to green calli.

TDZ at 1.0μM significantly increased shoot elongation and recorded the highest shoot length throughout the culture period. TDZ has been reported to induce morphogenic responses with efficiency comparable to or higher than that of other adenine containing cytokinins or a combination of auxin and cytokinin (Murthy *et al.*, 1998). The high efficiency attributed to TDZ may be as a result of TDZ acting directly as a cytokinin or through the modulation of endogenous growth substances (Hutchinson *et al.*, 1996a; Mok *et al.*, 1982). These findings support the observation that TDZ at 1.0μM had the highest shoot elongation throughout the culture period.

Increasing TDZ concentration to 5.0μM had a significantly negative effect on shoot elongation, resulting to inhibition of shoot elongation. Supra-optimal levels of TDZ have been reported to inhibit morphogenic responses: somatic embrogenesis in geranium (Hutchinson *et al.*, 1996a; Visser *et al.*, 1992), poor elongation of shoots (Murthy *et al.*, 1998), inhibition of shoot growth in oats, maize, radish etc (Devlin *et*

al., 1989), stunting of shoots and roots in peanut (Murthy *et al.*, 1995), shortening and thickening of the hypocotyl in pumpkin (Burkanova *et al.*, 1984).

The inhibition of shoot elongation by TDZ at a higher concentration of 5.0μM may be attributed to ethylene effects. TDZ has been reported to cause elevations in endogenous ethylene (Hutchinson *et al.*, 1997b). Ethylene in turn causes stem thickening and shortening (Salisbury and Ross, 1991). These results could explain the low increase in shoot elongation in medium supplemented with higher concentrations of TDZ at 5.0μM.

Addition of NAA to medium containing TDZ or BAP significantly inhibited shoot elongation and induced callus formation. Sanyal *et al.* (1998) induced callus from tuberose leaf explants using a combination of 0.2-0.5mg L^{-1} NAA and 2mg L^{-1} BA. Addition of NAA to TDZ has been reported to have no effect on somatic embryogenesis in geranium hypocotyl cultures (Visser *et al.*, 1992). Watad *et al.* (1996) also reported no significant difference in the regeneration of shoots in carnation between medium containing TDZ alone or in combination with NAA. Previous studies have not reported callus induction using a combination of TDZ+NAA. In the present study, a different morphogenic pathway was stimulated when NAA was added to TDZ. A similar effect was observed with BAP. We can speculate that TDZ acted in a similar fashion to BAP when NAA was included in the culture medium. The possibillity of TDZ acting as a cytokinin, and with much higher efficiency has been demonstrated in numerous studies (Capelle *et al.*, 1983; Mok *et al.*, 1982; Thomas and Katterman, 1986).

Medium without PGRs supported a slight increase in shoot elongation. Suggesting that the shoot tissues may have endogenous growth substances, which could have supported the slight increase in shoot elongation. These findings further

suggest that exogenously applied PGRs influence shoot elongation. Inclusion of NAA at 0.1 and 0.01mg L^{-1} in MS medium did not support the survival of shoot tip explants, which became necrotic and died. Previous studies have reported the survival of shoot-tip explants in medium amended with a combination of auxin and cytokinin (Rabinowich and Brewster, 1990). Probably this could be the reason why the shoot-tip explants died when placed on medium consisting of auxins alone.

Morphogenic responses included the development of single shoots and induction of friable to compact, cream to green callus from shoot-tip explants of tuberose cultures. However, multiple shoots were not observed in any of the treatments. These results suggest that tuberose exhibited a high apical dorminance, a phenomenon which is common to most monocotyledonous plants, as was reported in *Alstroemeria* (Bond and Alderson, 1993). Similarly, Salisbury and Ross (1991) reported that shoot-tip explants contain high levels of auxins, which favour the elongation of shoots as opposed to the multiplication of shoots. The development of multiple shoots has been shown to require an intricate balance of auxin and cytokinin in several plant systems (Kyte, 1991), and may be influenced by the type of explant used. Sanyal *et al.* (1998) reported multiple shoot regeneration from callus induced leaf explants of tuberose using a narrow range of BAP+NAA ratio (0.1-0.25).

In conclusion, TDZ at 1.0μM recorded the highest shoot elongation compared to a combination of BAP+NAA, which significantly inhibited shoot elongation and induced friable to compact, cream to green callus. These findings suggest that TDZ can be used to support the growth of tuberose shoot-tip explants, which could enable the production of clean plants, *in vitro* conservation of germplasm and exchange, to support crop improvement programmes.

Shoot quality rating

In the present study, treatments containing TDZ and BAP alone at various concentrations or in combination with NAA had a significant influence on the quality of shoots from 3 to 12 weeks in culture. Good quality shoots, which were characterised by healthy, light-green to green and vigorous growth were observed in most of the treatments except MSO medium in the 10^{th} and 12^{th} week in culture, and medium supplemented with a combination of TDZ+NAA or BAP+NAA during the first five weeks in culture.

Thidiazuron at (0.1, 0.4, 1.0, and 5.0μM) had no significant increase on the quality of shoots at 3 and 5 weeks in culture. Suggesting that the tissues may have been still utilizing endogenous growth substances to maintain high shoot quality. Also, there is a possibility of TDZ not being utilized immediately in tissues (Dysons, 1978; Mok and Mok, 1985). At 8 to 12 weeks in culture, TDZ at various concentrations significantly increased the quality of shoots. At this point, probably exogenously applied TDZ was utilized in tissues and significantly increased the quality of shoots.

During the 12^{th} week in culture, TDZ at 5.0μM supported significantly high quality of shoots compared to medium containing (0.1, 0.4, and 1.0) μM TDZ. Treatment with TDZ has been found to protect chlorophyll from degradation in detached leaves of barley (You *et al.*, 1992), and geranium tissues treated with TDZ had higher levels of chlorophyll (Visser *et al.*, 1995). Murthy *et al.* (1995), reported greening (high chlorophyll concentration), and thickening of cotyledons accompanied by stunting of shoots and roots in peanut treated with TDZ. In another study, TDZ was reported to act as a cytokinin by causing retention of chlorophyll (Mok *et al.*, 1982).

These findings support the evidence that TDZ could have increased shoot quality by increasing chlorophyll content and the effect was highest at 5.0μM, although other levels (0.1, 0.4, and 1.0) μM, supported satisfactory increase in shoot quality. In this regard, TDZ could have acted as a cytokinin by increasing the chlorophyll content. Cytokinins have been shown to stimulate the synthesis of proteins, some of which bind to chlorophyll and increase the content which ultimately could improve the quality of shoots (Mutui, 1999; Salisbury and Ross, 1991).

N^6 -Benzylaminopurine at 0.05, 0.1, and 1mg L^{-1} had no significant increase on the quality of shoots in the 3rd and 5th week in culture. This results suggest that endogenous growth substances may have still been actively used to maintain good quality of shoots, and or a time lag was required before the shoot tissues could respond to exogenously applied BAP (Salisbury and Ross, 1991). The treatment containing BAP at 0.1-1mg L^{-1} significantly increased shoot quality, which was significantly higher than BAP at 0.05mg L^{-1}. These results suggest that BAP at higher levels (0.1 and 1.0mg L^{-1}) could have had a tendency to concentrate chlorophyll content, which may have enhanced good quality shoots. In contrast, lower BAP levels (0.05)mg L^{-1} supported shoots with lower shoot quality perhaps as a result of lower concentration of chlorophyll content.

Medium containing a combination of TDZ+NAA and BAP+NAA had no significant increase on shoot quality in the first 5 weeks in culture, except medium containing 0.1μM TDZ+0.01mg L^{-1} NAA in the 3rd and 5th week in culture and 0.1mg L^{-1} BAP+0.01mg L^{-1} NAA in the 5th week, which significantly decreased the quality of shoots to (pale green- brown). Probably, as a result of the relatively low BAP (0.1mg L^{-1}) and TDZ (0.1μM) content. In addition, before callus induction, the shoot tissues had poor quality ranging from pale green to brown. Furthermore, the

concentration of PGRs used had more effect on shoot quality rather than the ratio used, with lower concentrations having a significant decrease on shoot quality. In addition, lowering BAP or TDZ levels in the presence of NAA may have shifted towards higher levels of NAA in the tissues, with a subsequent increase in ethylene production, which could have enhanced degradative processes (respiration, loss of membrane integrity, discolouration, and senescence) (Chebet, 1999; Mutui, 1999; Salisbury and Ross, 1991).

MSO medium supported significantly high shoot quality in the 3rd and 5th week in culture. These findings may suggest the influence of endogenous growth substances in increasing shoot quality. However, a decline in shoot quality was observed in subsequent weeks, probably because the endogenous growth substances had been depleted and there were no additional growth substances to sustain good quality of shoots. These results are similar to those observed when leaves were detached from intact bean (*Phaseolus vulgaris*) plants (Salisbury and Ross, 1991).

Based on findings aforementioned, TDZ may have acted as a cytokinin by influencing the concentration of chlorophyll content. However, the possibility of TDZ substituting for auxin and cytokinin requirement can not be ruled out, since most growth processes require an interaction of phytohormones (Hutchinson *et al.*, 1997a, 1997b). Similarly, the evidence that BAP acted in a similar fashion to TDZ by increasing chlorophyll content cannot be ignored.

In summary, TDZ caused a significant increase on the quality of shoots with increasing concentration compared to a combination of BAP+NAA, which had no significant increase on the quality of shoots, and most of the shoots were pale green to brown.

Mean number of leaves per shoot

Plant growth substances influence the development of buds, shoots, and leaves. Axillary bud development is influenced by cytokinins while apical buds cause shoot elongation under the influence of auxins (Salisbury and Ross, 1991).

A significant increase in the number of leaves was observed in medium containing relatively lower TDZ concentrations at 0.1, 0.4 and 1.0μM, from 3 to 12 weeks in culture. Again, there was a time lag before the tissues responded to the effect of TDZ in the medium, This could be attributed to the time lag required for TDZ to influence metabolic processes which may have subsequently influenced the growth and development of leaves. This can be supported in accordance to the view that TDZ persisted in tissues in a previous study on (*Phaseolus lunatus* L.) (Dysons, 1978).

From 3 to 10 weeks in culture, there was no difference in the number of leaves observed in medium containing TDZ at 0.1, 0.4, and 1.0μM. However, at 12 weeks in culture, TDZ at 1.0μM recorded a significantly higher increase in the number of leaves compared to medium containing 0.1, 0.4, and 5.0μM TDZ. According to related studies, TDZ has been reported to influence the regeneration of shoots with an efficiency comparable to or higher than that achieved by other phytohormones, and at relatively lower concentrations in several plant systems (Murthy *et al.*, 1998). Wang *et al.* (1986), reported the induction of bud break and development in apple treated with TDZ. Although satisfactory growth and development of leaves was also observed in TDZ at 0.1 and 0.4μM throughout the culture period, probably, 1.0μM TDZ provided optimum conditions for the growth and development of leaves in tuberose.

A high TDZ concentration of 5.0μM caused no significant increase in the number of leaves from 3 to 10 weeks in culture. However, a significant increase in the

number of leaves was observed at 12 weeks in culture which was comparable to that recorded in medium containing 0.1 and 0.4 μM TDZ and significantly lower than that observed in medium containing 1.0μM during the same period. The fewer number of leaves observed in medium containing 5.0μM TDZ can be attributed to the inhibition of shoot elongation, which may have resulted in fewer leaves. The number of leaves improved at 12 weeks in culture, at the time when also a considerable increase in shoot elongation was observed, as previously reported on the effect of TDZ at 5.0μM on shoot elongation

Medium containing BAP at 0.05, 0.1, and 1mg L^{-1} had no significant increase on the number of leaves from 3 to 8 weeks in culture. Probably, as a result of increased inhibition of shoot elongation which resulted in fewer leaves. However, from 10 to 12 weeks in culture, lower levels of BAP at 0.05mg L^{-1} recorded a significant increase in the number of leaves, though they were few and comparable to those recorded in medium containing 0.1 and 1.0mg L^{-1} BAP. In general, BAP at various concentrations supported fewer numbers of leaves and only achieved a satisfactory level in the 12^{th} week. This could be attributed to the increased inhibition in shoot elongation observed in medium containing BAP at various concentrations. BAP has been reported to influence shoot multiplication compared to shoot elongation (Salisbury and Ross, 1991; Economou *et al.*, 1986), subsequently, recording shorter shoots with fewer leaves.

A combination of TDZ+NAA and BAP+NAA in the medium recorded no significant increase in the number of leaves in the first 5 weeks in culture, and a decline in the number of leaves was observed from 8 to 12 weeks in culture. Probably as a result of changes in morphogenic responses which were achieved through a shift

from shoot elongation to inhibition through formation of friable to compact, cream to green callus.

Medium without any PGRs supported very few healthy, well developed leaves, and in most of the treatments, the first leaf did not open and olly slightly elongated during the growth period. Suggesting that proper growth and development of leaves is influenced by plant growth regulators (Salisbury and Ross, 1991; Skoog and Miller, 1957). Perhaps the slight increase in growth achieved was only influenced by endogenous growth substances which were not adequate to support substantial growth and development of leaves.

Finally, TDZ at 1.0μM supported the highest number of leaves compared to a combination of BAP+NAA throughout the culture period. A combination of BAP+NAA significantly inhibited the development of leaves, and induced friable to compact, cream to green callus. TDZ at higher levels (5.0μM) supported low number of leaves in the first 10 weeks in culture. Comparable results were observed in medium containing BAP at higher levels (0.1 and 1.0)mg L^{-1}. Suggesting that the low shoot elongation observed (*see* shoot length), could have also resulted in fewer number of leaves.

Bulblet formation

In addition to acting in a similar fashion to BAP, TDZ induced bulblets at 0.4 and 1.0uMTDZ (Fig.2). The induction of bulblets may be attributed to the ability of TDZ in regulating endogenous growth substances, some of which have been reported to be associated with stress and formation of organs that ensure survival of species. Such growth substances include ABA, GA and ethylene (Hutchinson ,1996; Salisbury and Ross, 1991). This could provide an alternative regeneration procedure that may be

more efficient in handling and storage similar to that of microtubers in potato (*Solanum tuberosum* L.) (Dodds, 1989).

Rooting

There are several factors which affect rooting in plants. They include; anatomical, physiological and environmental factors (Hartman, 1985; Janick, 1986; Salisbury and Ross, 1991). Physiological and anatomical effects on rooting are further influenced by genetic factors.

In the present study, physiological factors, especially rooting substances in plants were considered. MSO medium supported the growth of some roots, which were not significantly different from those observed in medium containing 1.0mg L^{-1} NAA; suggesting that plant tissues contain endogenous auxins in form of IAA which induce rooting (Salisbury and Ross, 1991). Medium supplemented with 0.1mg L^{-1} NAA recorded a significant increase in the mean number of roots, although it was not significantly different from that observed in medium containing 0.1mg L^{-1} IBA. These findings support the hypothesis that the number of roots was not dependent on the type of auxin used in the rooting medium. This further suggests a common induction site for the different types of auxins. However, the level of concentration within NAA had a significant difference in the number of roots induced, suggesting that higher levels of NAA (1.0mg L^{-1}) had no increase on the number of roots, probably even causing inhibition. This is consistent with previous studies on physiological responses induced by PGRs. Lower levels of PGRs are not usually adequate to support appreciable growth, while, supraoptimal levels inhibit growth (Janick, 1986). Sanyal *et al.* (1998), demonstrated that 0.1mg L^{-1} NAA induced better rooting in tuberose shoots. These results further support the observation that NAA at 0.1mg L^{-1} induced better rooting of tuberose shoots in the present study. In addition, results recorded in

medium containing 0.1mg L^{-1} IBA were not significantly different from those observed in MSO medium, suggesting lower efficiency.

Basal medium recorded a considerable increase in root length. These results could suggest the important role played by nutrient salts in the medium on root elongation (Preece, 1995). Indeed, addition of auxins to the rooting medium did not significantly increase root elongation. We could speculate that the presence of endogenous auxins inhibits the response to exogenously applied auxins, and that probably exogenous auxins may only be utilized once the endogenous supplies are depleted. Although, there was a significant difference in the mean root length between medium containing 0.1mg L^{-1} IBA and 1.0mg L^{-1} NAA, with better root elongation observed in medium containing 0.1mg L^{-1} IBA

Shoots with more roots tended to grow better and also acclimatised with ease, especially if the shoot also had a minimum of three leaves.

Effect of previous medium on rooting of Tuberose shoots in subsequent rooting medium

Indolebutiricacid (IBA) readily induces rooting. This was also observed in the rooting experiments where 80% of the explants rooted, although the mean number of roots was lower, but not significantly different from that recorded in medium containing 0.1mg L^{-1} NAA. This evidence was further supported by the ability of IBA to induce rooting in shoots previously grown in Medium containing BAP, an observation not recorded in other rooting media. Cytokinins have been reported to inhibit root formation in leaf explants (Kaul and Sabharwal, 1972; Paterson and Rost, 1981; Hutchinson *et al.*, 1996a). When placed in medium with or without other auxins (NAA), shoots previously grown in medium containing BAP did not root.

Shoots previously grown in medium supplemented with 1.0μM TDZ had the longest shoots with the highest number of leaves. Probably the availability of adequate leaves could have increased the carbohydrate supply to the root primodia. This together with availability of co-factors, may have enhanced rooting (Janick, 1986). The level of TDZ also could have played a crucial role, in that, at higher levels (5.0μM), only fair rooting was observed in medium containing 0.1mg L^{-1} NAA. However, at 1.0μM TDZ, optimum conditions might have been achieved to promote rooting when 0.1mg L^{-1} NAA was added. Higher levels of TDZ have been reported to induce accumulation of endogenous growth substances (Hutchinson *et al.*, 1996a), including: cytokinins, auxins, and ethylene, which may cause inhibition of rooting at high levels (Salisbury and Ross, 1991).

CHAPTER FOUR

The potential of using thidiazuron in *in-vitro* propagation of *Alstroemeria aurantiaca* cv. Rosita

Abstract:

Shoot regeneration was achieved in shoot-tip cultures of *Alstroemeria aurantiaca* cv. Rosita inoculated on media supplemented with various concentrations of thidiazuron (TDZ). TDZ at 0.1 to 1μM induced shoot regeneration with efficiency comparable to that obtained by conventionally used combination of BAP and NAA. However these results were obtained in the last week of culture, suggesting TDZ alone not to be a chemical of choice for the in vitro propagation of *Alstroemeria aurantiaca* cv. Rosita.

Introduction

Alstroemeria were among the first plants of the genera *Alstroemeria* to be brought into Europe from South America by a Swedish botanist called Baron Klas van Alstroemeria and *Alstroemeria spp* were named after him (HCDA, 1996). Peru, in South America has been reported as the centre of origin of about 60 *Alstroemeria* species (Uphof, 1952). *Alstroemeria* species, along with it's improved cultivars and hybrids have been widely grown as cut flowers. This is partly due to it's ease of management as an outdoor crop, high yield and consumer interest (Healy and Wilkins, 1979). Intensive breeding has been done on the crop, and the improved varieties are subjected to plant breeders' rights (Heins and Wilkins, 1979). Consequently, growers have to pay for royalties, making the planting materials expensive (Mutui, 1999).

The white settlers are thought to have introduced the crop to Kenya in the late 60's and early 70's (Chepkairor, 1986). Several varieties of this crop are grown in Kenya, and currently, new varieties include; "Yellow King", "Jubilee", and "Rosita" (HCDA, 1996). The crop does well in areas with cooler soil temperatures (10-15°C). The low soil temperatures are required for flower induction on the rhizomes.

In Kenya, the crop is grown in upper Kiambu and the following areas are also suitable; Kinangop, Kericho, Molo, Kaptagat, Wundanyi, Timboroa and Meru (HCDA, 1996; Mutui, 1999).

Alstroemeria belongs to the family *Alstroemeriaceae*, formerly *Amaryllidaceae* (Uphof, 1952). It is a herbaceous perennial, which produces tall slender leafy stems up to 2 meters high. It terminates with red, pink, purple, yellow or white flowers (Uphof, 1952; HCDA, 1996). Most of the available commercial varieties are interspecific hybrids from the following species; *Alstroemeria aurantiaca* D. Don ex Sweet, *Alstroemeria violocea* phil, *Alstroemeria haemantha* Ruitz pav., *Alstroemeria ligtu* L., *Alstroemeria pelegrina* L., and *Alstroemeria Pulchra* Sims (Herns and Wilkins, 1979).

Aerial shoots arise from an underground sympodial rhizome (Heins and Wilkins, 1979). The rhizome apex develops from an axillary bud of the first scale leaf of the previous shoot (Buxbaum, 1951). The second axilliary bud has the potential to become a lateral rhizome. Aerial shoots can be vegetative or generative depending on the environmental conditions (Heins and Wilkins, 1979). The crop has a fibrous root system, which can be fine or thick and fleshy. The leaves have parallel veins and are twisted at the junction of the sheath and the blade causing the leaf to be inverted 180°C (Priestly *et al.*, 1935). Stomates on the adaxial surface are also inverted (Heins and Wilkins, 1979).

The inflorescence is a whorl of simple or compound cymes (Whyte, 1929) which form a terminal branched cluster of flowers. Each cyme can consist of several flowers arising sympodially. The perianth segments are separeted. These could be equal or unequal. It has 6 stamens, which are delineated. The ovary is inferior, 3-celled and develops into a capsule (Healy and Wilkins, 1985).

Most of *Alstroemeria* varieties are sterile hybrids, hence they are propagated by divition of underground rhizomes with attached roots (Healy and Wilkins, 1985) or tissue culture (Ziv *et al.*, 1973). In Kenya, planting is done in April/May to produce flowers for export in September or December (HCDA, 1996).

The potential yield and quality of *Alstroemeria* has not been realised in the past years, this is mainly due to farmers using infected rhizome divisions (HCDA,1997). The propagules are also slow to multiply using conventional methods, as a result, clean planting materials are not readily available. Although seed propagation can be done, propagation by seed is generally avoided due to genetic variability. A few medium and large-scale farmers import the planting materials. However, this has proved to be expensive due to high charges on royalties and breeders' rights (Mutui, 1999)

Alstroemeria is susceptible to a number of viruses (Potyviruses and Tomato spotted wilt virus) and fungal infections (root rot and botrytis caused by *Pythium* and *Rhizoctonia*) (Bridgen *et al.*, 1993). Although the planting material can be drenched in fungicides, over-dependence on chemicals is detrimental to the environment (HCDA, 1996). Moreover, viruses are not eliminated and some fungi develop resistance to the chemicals with time (KARI, 1993).

Fortunately, the use of tissue culture techniques can consistently produce virus free and disease free plants (Kartha, 1986; Krikorian, 1982). This can be achieved if

efficient protocols are developed (Kitto, 1997). One way of developing efficient protocols is through the use of potent plant growth regulators such as thidiazuron (Hutchinson *et al.*, 1996a ; Murthy *et al.*, 1998; Visser *et al.*, 1992). This is usually a faster, cheaper and simple method compared to those involving the manipulation of regeneration pathways and nutrient media. Previously, a combination of auxin and cytokinin based on the Skoog and Miller hypothesis (1957), has been used to induce morphogenic responses in plants. Bridgen *et al.* (1992), demonstrated the multiplication of rhizomes in *Alstroemeria* hybrids using a combination of 1-5mg L^{-1} BAP and 0.01mg L^{-1} NAA. Similarly, Gabrezuesker (1995), used a combination of 2mg L^{-1} BAP and 0.5mg L^{-1} NAA to regenerate shoots from shoot-tip explants of an *Alstroemeria* spp.

Thidiazuron, a substituted phenylurea (N'-phenyl-N'-1,2,3-thidiazol-5-ylurea, TDZ), which was primarily used as a cotton defoliant (Arndt, 1996), has been shown to mimic cytokinin activity in inducing morphogenic responses with an efficiency similar to or higher than that of N^6 – substituted adenine derivatives (Mok *et al.*, 1982). In addition, TDZ substituted for auxin and cytokinin requirement with an even greater efficiency in the development of somatic embryos in geranium hypocotyl cultures (Hutchinson *et al.*, 1996a; Visser *et al.*, 1992), and Neem (*Azadirachta indica* A. Juss) (Murthy and Saxena, 1998).

A few authors have reported studies on *Alstroemeria* using TDZ for *in-vitro* plantlet regeneration. Lin *et al.* (1997), obtained the best direct shoot regeneration from excised leaf explants of in-vitro grown seedlings of *Alstroemeria* L., with TDZ at 10μM and IBA at 0.5μM in the induction medium, and BAP at 2.2μM in the regeneration medium. Similarly, Hutchinson et al. (1994) reported induction of multiple shoots from callus induced from mature zygotic embryos of a tetraploid

Alstroemeria (A. *pelegrina* x A. *psittacina*), using TDZ (0.5μM) in combination with BAP (8μM). To our knowledge, there are no studies reported on TDZ-mediated regeneration of shoots from *Alstroemeria* cv. Rosita shoot-tip explants, in order to provide clean planting materials. In this study, I demonstrate the possibility of TDZ substituting for a combination of auxin and cytokinin requirement in morphogenic responses and plant regeneration of *Alstroemeria* cv. Rosta apical shoot-tip cultures.

The main objective of this part of the study was to determine the role of TDZ in morphogenesis and plant regeneration in *Alstroemeria* shoot-tip cultures.

The specific objectives were:

(i) To investigate the effect of TDZ compared to a combination of BAP and NAA on morphogenesis and regeneration of plantlets from *Alstroemeria* shoot-tip cultures.

(ii) To determine the effect of IBA on rooting of *Alstroemeria* shoots previously cultured in medium containing TDZ at various concentrations, or a combination of BAP and NAA.

Materials and Methods

Site

Alstroemeria aurantiaca cv. 'Rosita' stock plants with both shoots and rhizomes were obtained from KARI, Tigoni research farm in Limuru. Limuru is at an altitude of 1800-2100m. above sea level. The area experiences a bimodal distribution of rainfall, with long rains in March-June and short rains in October-December. The annual rainfall is between 700-1000mm. The mean maximum day temperature is about 23°C and mean minimum of 15°C.

Plant Material

Alstroemeria was grown under outdoor conditions with minimal supplemental irrigation during the dry seasons (January-March) and (July-September). Rhizomes were dipped in benlate before planting at 10-15cm deep, on 1m wide raised beds. During land preparation, the soil was incorporated with TSP at 500kg/ha. The rhizomes were planted at a spacing of 35-40cm between rows and 40-50cm within rows, making 2 rows per 1m wide bed. Manual weeding was done to keep the beds weed free. The crop was sprayed against fungal infections (stem rot, botrytis e.t.c.), and spider mites using Dithane M45 and Metasystox, respectively. Top dressing with NPK (17-17-17) and CAN in the ratio of 1:1 at a rate of 2Kg/100 metre of bed length was done every two weeks during the cropping seasons, as recommended in manual for floriculture (KARI, 1996).

Preparation of explant and sterilisation

Alstroemeria aurantiaca cv. Rosita plants were obtained from 3 months old crop, during the month of February towards the end of the dormancy period (November/February). The rhizomes were cleaned with detergent (Bioagent) and rinsed in running tap water for 15 minutes. Excised tips (1-2cm long) from the rhizomes were placed in tap water in a beaker. The water was drained and the tips were immersed for 5 minutes in 95% alcohol. The alcohol was drained and the tips were rinsed in sterile distilled water for 3 minutes. The tips were placed in 0.5% NaOCl containing 'Tween 20' (2 drops/ 100mL solution), for 20 minutes, washed in three changes of distilled water and placed in a dry sterile petri dish. Each subsequent transfer of shoot-tips to alcohol, NaOCl, or sterile distilled water, was accompanied by agitation of the beaker through slight shaking by hand. Shoot-tips (0.5-1mm long),

consisting of an apical dome and one to two leaf primodia were excised under a dissecting microscope and inoculated.

Culture of shoot-tip explants

Excised shoot-tips were cultured, a single explant per universal bottle containing 10mL of medium. The medium consisted of MS (Murashige and skoog, 1962) salts, B5 (Gamborg *et al.*, 1968) vitamins, 30g. L^{-1} sucrose, 8g.L^{-1} agar, and different types and concentrations of PGRs. The BAP+NAA complement used in the present study, was found suitable for shoot regeneration from *Alstroemeria* spp. (Bridgen *et al.*, 1992; Gabryszewska, 1995). The concentration of BAP and NAA used are based on a previously published protocol (Bridgen *et al.*, 1992), which gave optimum results during preliminary studies.

A total of 13 treatments, each replicated four times and arranged in a completely randomised design (CRD) were used as follows:

1. MSO
2. 0.1μM TDZ
3. 0.4μM TDZ
4. 1.0μM TDZ
5. 5.0μM TDZ
6. 1mg L^{-1} BAP+0.01mg L^{-1} NAA
7. 1mg L^{-1} BAP+0.1mg L^{-1} NAA
8. 1 μM TDZ+0.01mg L^{-1} NAA
9. 1 μM TDZ+0.1mg L^{-1} NAA
10. 0.01mg L^{-1} NAA
11. 0.1mg L^{-1}NAA
12. 0.1mg L^{-1}BAP
13. 1.0mg L^{-1}BAP

Preliminary studies using 0.1, 1.0, and 5.0μM TDZ and a duration of exposure for 3, 10, and 15 d, showed that an exposure for 10 days at 1.0μM had more shoot-tips surviving after 5 to 7 d of inoculation, based on the colour of the shoot-tips (green-best surviving, light green to yellow-fair survival, or brown-dead). A duration of exposure for 10 d was also consistent with that used by Lin *et al.* (1997). Consequently, the shoot-tips were exposed to TDZ alone or in combination with NAA for 10 days before they were transferred to MSO medium. Subculturing was done after every four weeks. At the end of the 16 weeks in culture, the shoots were

transferred to rooting medium consisting of 3mg/l IBA (Gabryszewska, 1995). Rooting was scored after 4 weeks subculture, based on the number of cultures in a treatment initiating roots at the base of the shoot: - = no rooting; + = 1 out of four cultures rooted; ++ = 2 out of four cultures rooted; +++ = 3 out of four cultures rooted; ++++ = all four cultures rooted. The pH of all media was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 20min. The cultures were placed on shelves set at 25° ± 2°C and illuminated (16-hour photoperiod; 70-78 μmol $m^{-2}s^{-1}$) by cool fluorescent tubes.

Measurements on number of shoots, shoot quality rating, number of leaves, and shoot length were recorded after every two weeks for four months. Shoot quality rating was scored on a 1-4 scale, based on visual attributes of colour (indicated by the level of chlorophyll content in the shoot tissues) and growth of shoots: 1 = brown, poor growth; 2 = pale green, severe chlorosis, fair to poor growth; 3 = light green, slight chlorosis, good growth; 4 = green, healthy and vigorous growth.

Statistical analysis

In this experiment, the treatments consisted of four replications and the experiment was repeated at least twice. Data were analysed using analysis of variance (GENSTAT statistical software (Lane and Payne, 1996), and the means were compared by Tukey's procedure at 5% level of probability.

Results

Number of shoots per explant

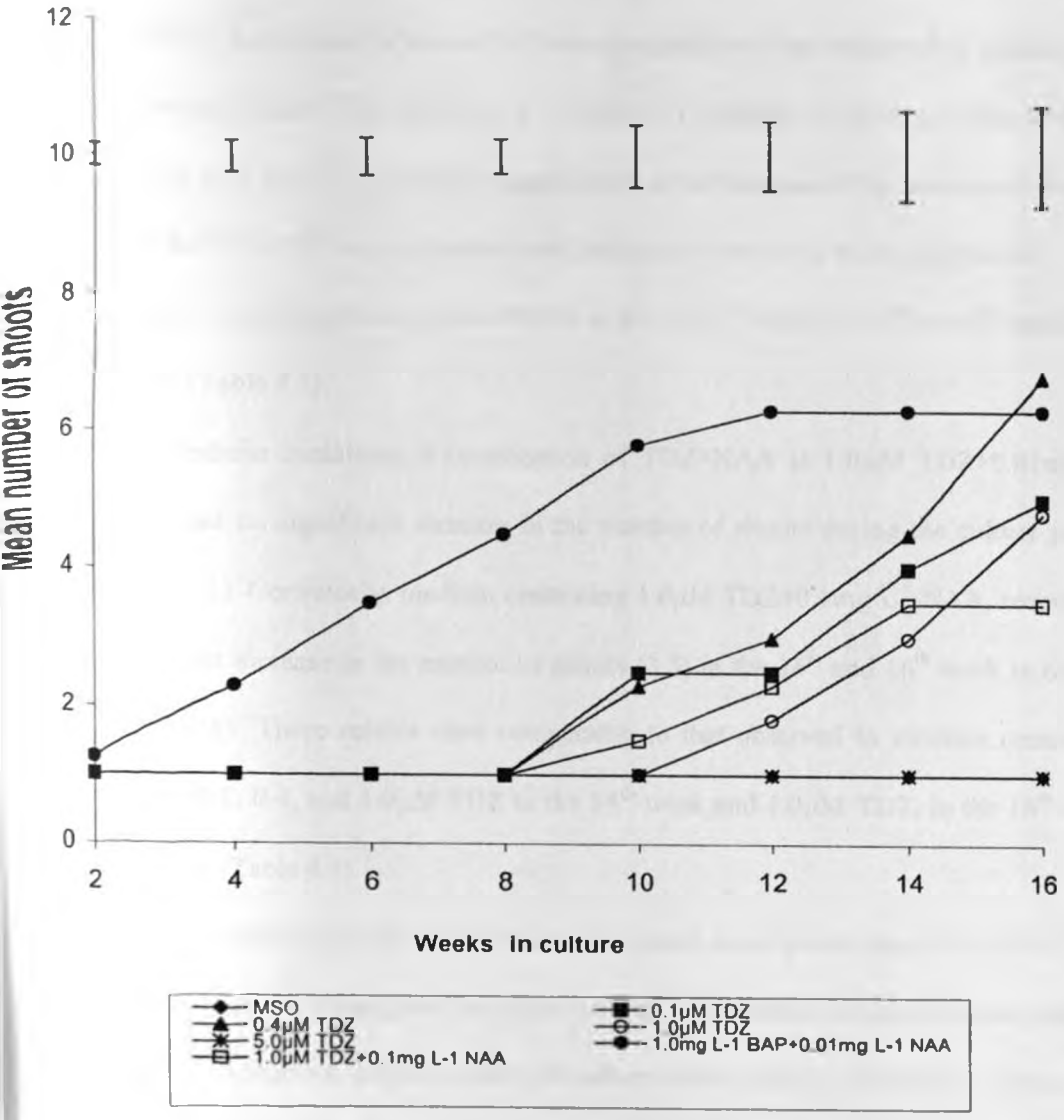
Analysis of variance revealed highly significant differences ($P \leq 0.001$) among the PGRs for the number of shoots from the 2nd to 16th week of culture (Table 4.1). Data for medium supplemented with 0.01 and 0.1mg L^{-1} NAA, and 0.1 and 1.0mg L^{-1} BAP alone was not included in the analysis, since the shoot-tip explants became necrotic and died after 7 d of culture. Medium containing a combination of BAP and NAA, maintained an increasing number of shoots from 2 to 12 weeks in culture after which there was no further increase in the number of shoots (Fig. 4.1). Medium supplemented with TDZ alone or in combination with NAA recorded an increase in the number of shoots after 8 weeks in culture (Fig. 4.1, Table 4.1). A high TDZ concentration of 5.0μM did not have a significant increase on the number of shoots, and maintained single shoots throughout the culture period (Fig. 4.1). Similar results were observed in MSO medium (Fig. 4.1, Table 4.1). TDZ at 0.1, 0.4, and 1.0μM had a significant increase ($P \leq 0.001$) in the number of shoots over the control at 16 weeks in culture (Fig. 4.1, Table 4.1). During the same culture period, TDZ at 0.4μM had a significant ($P \leq 0.001$) and highest number of shoots (6.8), compared to the rest of the medium containing TDZ at various concentrations (Fig. 4.1, Table 4.1). Among the TDZ treatments, medium containing 5.0μM recorded the lowest number of shoots (1) from 12 to 16 weeks in culture (Fig. 4.1).

A combination of BAP and NAA in the medium significantly increased ($P \leq 0.001$) the number of shoots relative to the control throughout the culture period (Fig. 4.1, Table 4.1). In addition, this same medium at 1.0mg L^{-1} BAP+0.01mg L^{-1} NAA,

Table. 4.1 Effect of TDZ at various concentrations alone or in combination with NAA on the mean number of shoots compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* cv. Rosita cultures

PGRs	Weeks in culture							
	2	4	6	8	10	12	14	16
MSO	1	1b[y]	1c	1c	1d	1c	1e	1e
0.1µM TDZ	1	1b	1c	1c	2.5c	2.5b	4bc	5b
0.4µM TDZ	1	1b	1c	1c	2.3c	3b	4.5bc	6.8a
1.0µM TDZ	1	1b	1c	1c	1d	1.8bc	3cd	4.8bc
5.0µM TDZ	1	1b	1c	1c	1d	1c	1e	1e
1.0mg L^{-1} BAP+0.01mg L^{-1} NAA	1.25	2.3a	3.5a	4.5a	5.8a	6.3a	6.3a	6.3ab
1.0mg L^{-1} BAP+0.1mg L^{-1} NAA	1	1.8a	2.5b	2.75b	4b	5a	5ab	5b
1.0µM TDZ+0.01mg L^{-1} NAA	1	1b	1c	1c	1d	1c	1.8de	2.5de
1.0µM TDZ+0.1mg L^{-1} NAA	1	1b	1c	1c	1.5cd	2.3bc	3.5bc	3.5cd
Significance	n.s	***	***	***	***	***	***	***
W(Turkey's)	0.39	0.53	0.63	0.59	1.1	1.39	1.55	1.72
SE	0.166	0.23	0.27	0.25	0.45	0.5	0.65	0.72
CV	16.2	19.3	18.1	16.1	20.0	20.0	19.6	18.3

,* =significant at $P \leq 0.01$, or 0.001 respectively.
[y] = Mean separation using Turkey's. Values having the same letters within the same column are not significantly different at 5% level of probability



– 1= mg in a liter

1 Effect of TDZ at various concentrations alone or in combination with NAA on the mean er of shoots compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* cv. a shoot-tip cultures. Vertical bars represent standard error.

recorded the highest number of shoots (2.3, 3.5, 4.5, 5.8, 6.3, and 6.3) at (4, 6, 8, 10, 12, and 14) weeks in culture, respectively (Fig. 4.1, Table 4.1). However, at 16 weeks in culture, the number of shoots (6.3) was comparable to that observed in medium containing 0.4μM TDZ (6.8) (Fig. 4.1, Table 4.1). Medium containing higher levels of NAA at 0.1mg L^{-1}, recorded a significantly lower increase in the number of shoots from the 6^{th} to 10^{th} week in culture, and had similar results to those observed in medium containing lower levels of NAA at 0.01mg L^{-1} from the 12^{th} to 16^{th} week in culture (Table 4.1).

Medium containing a combination of TDZ+NAA at 1.0μM TDZ+0.01mg L^{-1} NAA, had no significant increase in the number of shoots during the culture period (Table 4.1). Conversely, medium containing 1.0μM TDZ+0.1mg L^{-1} NAA, recorded a significant increase in the number of shoots (3.5) in the 14^{th} and 16^{th} week in culture (Table 4.1). These results were comparable to that observed in medium containing TDZ at 0.1, 0.4, and 1.0μM TDZ in the 14^{th} week and 1.0μM TDZ, in the 16^{th} week in culture (Table 4.1).

A combination of BAP and NAA supported a significantly high ($P \leq 0.001$) number of shoots throughout the culture period. The highest number of shoots was observed in medium supplemented with lower levels of NAA (0.01mg L^{-1}) (Table 4.1). TDZ at 0.4μM had comparable results to that observed in medium containing a combination of BAP and NAA at 16 weeks in culture (Table 4.1).

Number of leaves per shoot

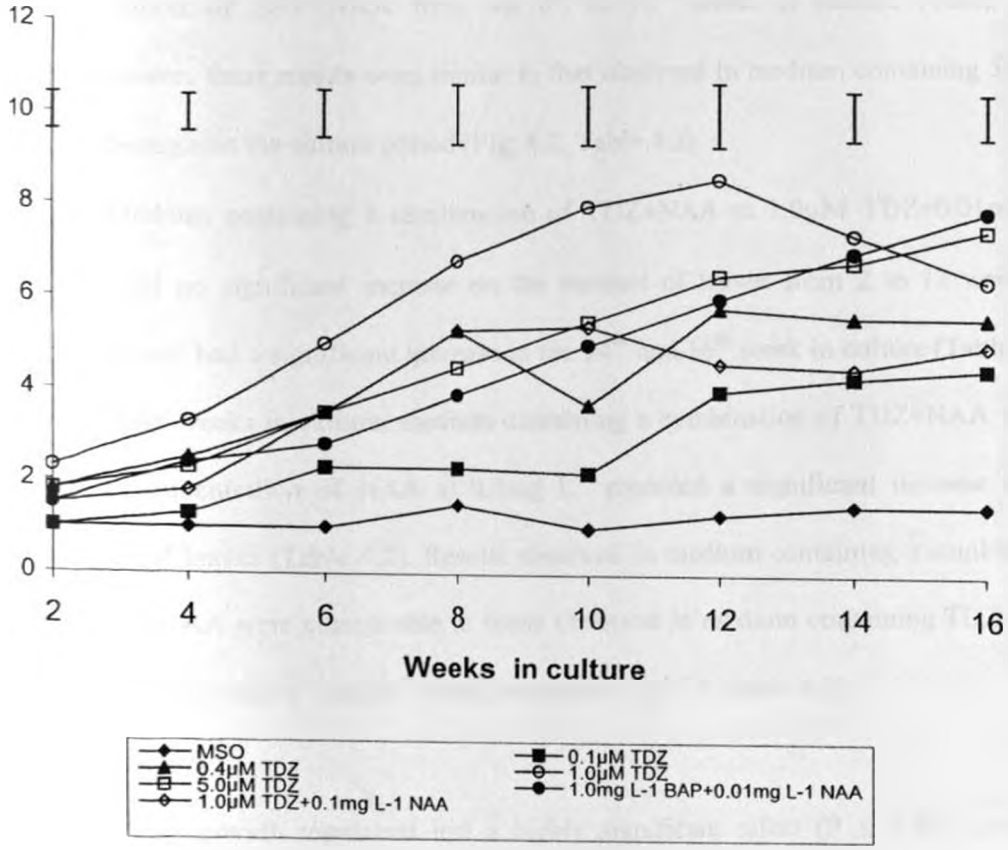
Analysis of variance revealed highly significant differences among PGRs for the number of leaves throughout the culture period (Table 4.2). An increase in the number of leaves was observed in all the treatments, except MSO medium up to 12 weeks in culture (Fig. 4.2). Notably, from 12 to 16 weeks in culture, medium containing 1.0μM TDZ recorded a decline in the mean number of leaves (Fig. 4.2, Table 4.2). Basal medium recorded very few healthy, visible, well-developed leaves ranging from (1to 1.5) during the culture period (Fig. 4.2, Table 4.2). TDZ at 0.1μM had no significant increase on the number of leaves during most of the culture period except the 10^{th} and 16^{th} week in culture, when a significant increase ($P \leq 0.001$) was noted (Table 4.2). Medium containing 0.4μM TDZ recorded a significant increase ($P \leq 0.001$) on the number of leaves from the 4^{th} week in culture (Fig. 4.2, Table 4.2). However, there was no significant difference in the mean number of leaves between medium containing 0.1μM TDZ and 0.4μM TDZ from 10 to 16 weeks in culture (Table 4.2).

TDZ at 1.0 and 5.0μM had a significant increase ($P \leq 0.001$) on the number of leaves over the control, from the 4^{th} to 16^{th} week in culture (Fig. 4.2, Table 4.2). Medium containing 1.0μM TDZ had a general increase in the number of leaves from the 2^{nd} to 12^{th} week in culture, after which a decline in the number of leaves was observed up to the 16^{th} week (Fig. 4.2, Table 4.2). TDZ at 1.0μM recorded a significantly higher number of leaves (3.3, 5, 6.8, 8, and 8.6) at (4, 6, 8, 10, and 12) weeks in culture, respectively compared to medium containing 5.0μM TDZ (Fig. 4.2, Table 4.2). At 14 and 16 weeks in culture, medium containing 5.0μM TDZ recorded a significantly high number of leaves (6.8 and 7.5) respectively, which was similar to that observed in medium containing 1.0μM TDZ (Fig. 4.2, Table 4.2).

Table. 4.2 Effect of TDZ at various concentrations alone or in combination with NAA on the mean number of leaves per shoot compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* cv. Rosita cultures

PGRs	Weeks in culture							
	2	4	6	8	10	12	14	16
MSO	1b[Y]	1d	1f	1.5c	1e	1.3e	1.5e	1.5f
0.1μM TDZ	1b	1.3cd	2.3de	2.3c	2.2de	4cd	4.3cd	4.5de
0.4μM TDZ	1.8ab	2.5ab	3.5bc	5.3ab	3.7cd	5.8bc	5.6bc	5.6cd
1.0μM TDZ	2.3a	3.3ab	5a	6.8a	8a	8.6a	7.4a	6.4bc
5.0μM TDZ	1.8ab	2.3bc	3.5bc	4.5b	5.5b	6.5b	6.8ab	7.5ab
1.0mg L^{-1} BAP+0.01mg L^{-1} NAA	1.5ab	2.4ab	2.8cd	3.9b	5bc	6b	7a	7.9a
1.0mg L^{-1} BAP+0.1mg L^{-1} NAA	1.5ab	3.4a	4.3ab	4.6b	4.8bc	5.5bc	6.6ab	7.6a
1.0μM TDZ+0.01mg L^{-1} NAA	1b	1d	1.3ef	1.3c	1.5e	2.5de	3.87d	3.7e
1.0μM TDZ+0.1mg L^{-1} NAA	1.5ab	1.8cd	3.5bc	5.3ab	5.4b	4.6c	4.5cd	5d
Significance	**	***	***	***	***	***	***	***
W(Turkey's)	1	1	1.24	1.59	1.55	1.84	1.3	1.17
SE	0.4	0.4	0.52	0.66	0.65	0.7	0.54	0.49
CV	21.2	21.4	17.5	17.1	15.9	15.5	10.4	9.0

,* =significant at $P \leq 0.01$ and 0.001 respectively.
Y = Mean separation using Turkey's. Values having the same letters within the same column are not significantly different at 5% level of probability



L-1= mg in a liter

4.2 Effect of TDZ at various concentrations alone or in combination with NAA on the mean number of leaves compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* Rosita shoot-tip cultures

Medium containing a combination of BAP+NAA recorded a significant increase ($P \leq 0.001$) on the number of leaves from the 4th week in culture (Table 4.2). There were no significant differences on the number of leaves between media containing a combination of BAP+NAA from the 6th to 16th week in culture (Table 4.2). Furthermore, these results were similar to that observed in medium containing 5.0μM TDZ throughout the culture period (Fig. 4.2, Table 4.2).

Medium containing a combination of TDZ+NAA at 1.0μM TDZ+0.01mg L^{-1} NAA had no significant increase on the number of leaves from 2 to 12 weeks in culture, and had a significant increase in the 14th and 16th week in culture (Table 4.2). After four weeks in culture, medium containing a combination of TDZ+NAA with a higher concentration of NAA at 0.1mg L^{-1} recorded a significant increase in the number of leaves (Table 4.2). Results observed in medium containing a combination of TDZ+NAA were comparable to those observed in medium containing TDZ at 0.1 and 0.4μM in the 14th and 16th week in culture (Fig. 4.2, Table 4.2).

Shoot length

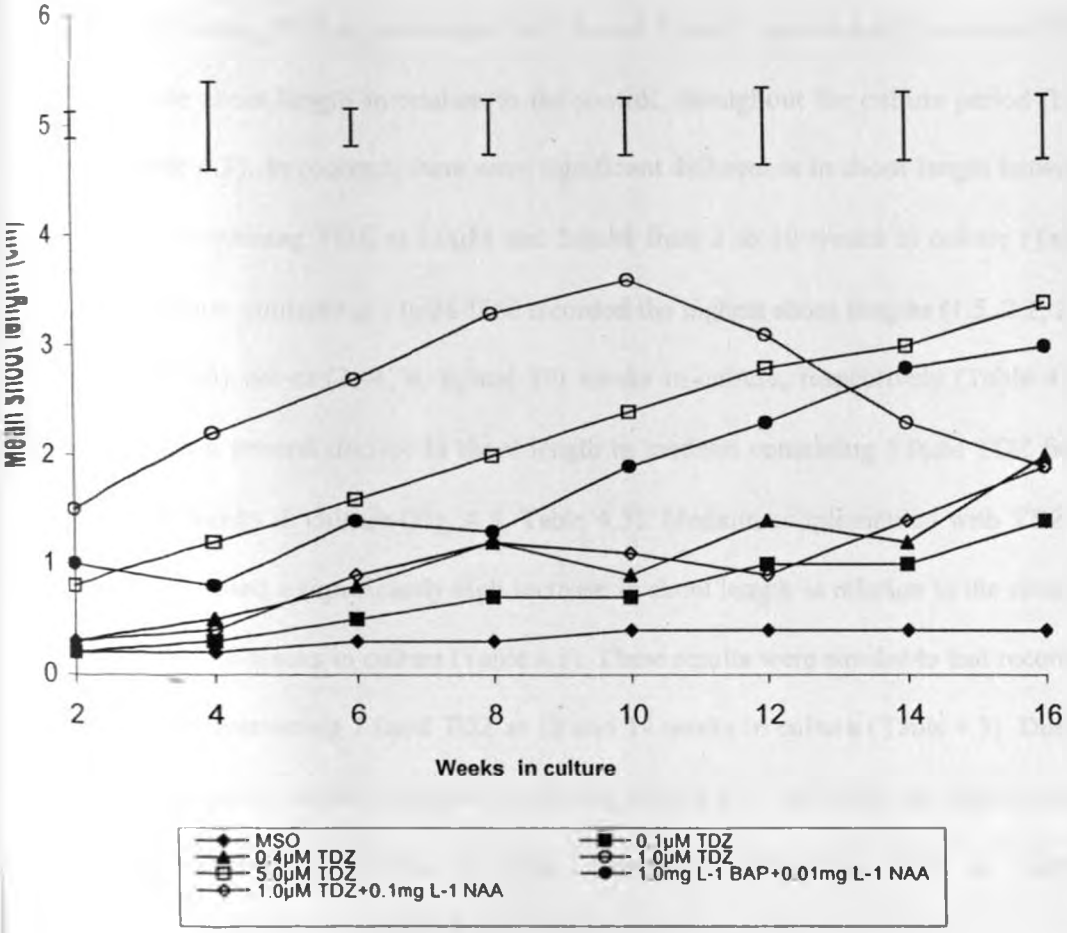
Plant growth regulators had a highly significant effect ($P \leq 0.001$) on shoot elongation (Table 4.3). An increase in shoot length was observed in most of the treatments except MSO medium from 2 to 10 weeks in culture (Fig. 4.3). A decline in shoot length was noted in medium containing 1.0μM TDZ after 10 weeks in culture (Fig. 4.3). Basal medium recorded low shoot lengths (0.2, 0.2, 0.3, 0.3, 0.4, 0.4, 0.4, and 0.4)cm at (2, 4, 6, 8, 10, 12, 14 and 16) weeks in culture, respectively (Fig. 4.3, Table 4.3). A low TDZ concentration of 0.1μM had no significant increase on shoot elongation over the control, from the 2nd to 14th week in culture (Fig. 4.3, Table 4.3). TDZ at 0.4μM had no significant increase on shoot length during the first 4 weeks in

Table. 4.3 Effect of TDZ at various concentrations alone or in combination with NAA on the mean shoot length (cm) compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* cv. Rosita shoot-tip cultures

PGRs	Weeks in culture							
	2	4	6	8	10	12	14	16
MSO	0.2c[y]	0.2d	0.3d	0.3d	0.4e	0.4e	0.4c	0.4d
0.1μM TDZ	0.2c	0.3d	0.5cd	0.7cd	0.7cd	1de	1bc	1.4bc
0.4μM TDZ	0.3c	0.5cd	0.8c	1.2c	0.9cd	1.4cd	1.2b	2b
1.0μM TDZ	1.5a	2.2a	2.7a	3.3a	3.6a	3.1a	2.3a	1.9b
5.0μM TDZ	0.8b	1.2b	1.6b	2b	2.4b	2.8ab	3a	3.4a
1.0mg L^{-1} BAP+0.01mg L^{-1} NAA	1b	0.8c	1.4b	1.3c	1.9b	2.3ab	2.8a	3a
1.0mg L^{-1} BAP+0.1mg L^{-1} NAA	1.3a	1.3b	1.6b	2b	2b	2bc	2.6a	2.8a
1.0μM TDZ+0.01mg L^{-1} NAA	0.2c	0.3d	0.3d	0.4d	0.6cd	1de	1bc	1.2c
1.0μM TDZ+0.1mg L^{-1} NAA	0.3c	0.4cd	0.9c	1.2c	1.1c	0.9de	1.4b	1.9b
Significance	***	***	***	***	***	***	***	***
W(Turkey's)	0.28	0.34	0.4	0.59	0.59	0.85	0.73	0.7
SE	0.12	0.4	0.17	0.25	0.26	0.35	0.31	0.29
CV	20.0	18	15.6	18.1	17.3	21.0	17.7	14.8

*** =significant at $P \leq 0.001$.

[y] = Mean separation using Turkey's. Values having the same letters within the same column are not significantly different at 5% level of probability



L-1= mg in a liter

4.3 Effect of TDZ at various concentrations alone or in combination with NAA on the mean oot length (cm) compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* cv. sita shoot-tip cultures. Vertical bars represent standard error.

culture compared to MSO medium (Table 4.3). However, a significant increase was noticed relative to the control, from 6 to 16 weeks in culture (Fig. 4.3, Table 4.3). Interestingly, there were no significant differences in shoot length between medium containing 0.1μM TDZ and 0.4μM TDZ (Fig. 4.3, Table 4.3).

Increasing TDZ concentration to (1.0 and 5.0μM), significantly increased ($P \leq 0.001$) the shoot length in relation to the control, throughout the culture period (Fig. 4.3, Table 4.3). In contrast, there were significant differences in shoot length between medium containing TDZ at 1.0μM and 5.0μM from 2 to 10 weeks in culture (Table 4.3). Medium containing 1.0μM TDZ recorded the highest shoot lengths (1.5, 2.2, 2.7, 3.3, and 3.6) cm at (2, 4, 6, 8, and 10) weeks in culture, respectively (Table 4.3). There was a general decline in shoot length in medium containing 1.0μM TDZ from 12 to 16 weeks in culture (Fig. 4.3, Table 4.3). Medium supplemented with TDZ at 5.0μM recorded a significantly high increase in shoot length in relation to the control, from 12 to 16 weeks in culture (Table 4.3). These results were similar to that recorded in medium containing 1.0μM TDZ at 12 and 14 weeks in culture (Table 4.3). During the 16th week in culture, medium containing 5.0μM TDZ recorded the highest shoot length (3.4cm) in relation to other treatments containing TDZ at various concentrations (Fig. 4.3, Table 4.3).

A significant increase in shoot length over the control was observed in medium containing a combination of BAP+NAA during the culture period (Fig. 4.3, Table 4.3). From 10 to 16 weeks in culture, there were no significant differences in shoot length between treatments containing NAA at 0.01mg L^{-1} and 0.1mg L^{-1} (Table 4.3). These results were similar to that observed in medium containing 5.0μM TDZ from 10 to 16 weeks in culture (Fig. 4.4, Table 4.3).

A combination of TDZ+NAA with lower NAA at 0.01mg L^{-1} had no significant increase on shoot length over the control, except in the 10^{th} and 16^{th} week in culture (Table 4.3). Medium containing higher NAA at 0.1mg L^{-1} recorded a significant increase in shoot length at 6, 8, 10, 14, and 16 weeks in culture, although in the 2^{nd} ,4^{th} and 12^{th} weeks, there was no significant increase in shoot length compared to MSO medium (Table 4.3). These results were comparable to those observed in medium containing 0.1 and 0.4μM TDZ (Table 4.3). Media containing a combination of TDZ+NAA had no significant differences in shoot elongation during most of the weeks (2, 4, 10, 12, and 14) (Table 4.3).

TDZ at 1.0μM recorded the highest shoot elongation during the first12 weeks in culture. However, in the last 14 to 16 weeks in culture, TDZ at 5.0μM recorded the highest shoot length. These results were comparable to those observed in medium supplemented with a combination of BAP and NAA during the last 14 to 16 weeks in culture.

Shoot quality rating

Analysis of variance detected highly significant differences ($P \leq 0.001$) for shoot quality rating among PGRs (Table 4.4). A general decline in the quality of shoots was observed in most of the treatments, except medium containing a combination of BAP+NAA and TDZ at (0.1-0.4 μM) during 12 to 16 weeks in culture (Fig. 4.4). MSO recorded the lowest shoot quality (1), which was observed as browning of shoots from 8 to 16 weeks in culture (Fig. 4.4, Table 4.4).

TDZ at 0.1, 0.4, 1.0, and 5.0μM had no significant increase on the shoot quality rating relative to the control, in the first 4 weeks in culture (Fig. 4.4, Table 4.4). In addition, there was a decline in the shoot quality rating in medium containing TDZ at

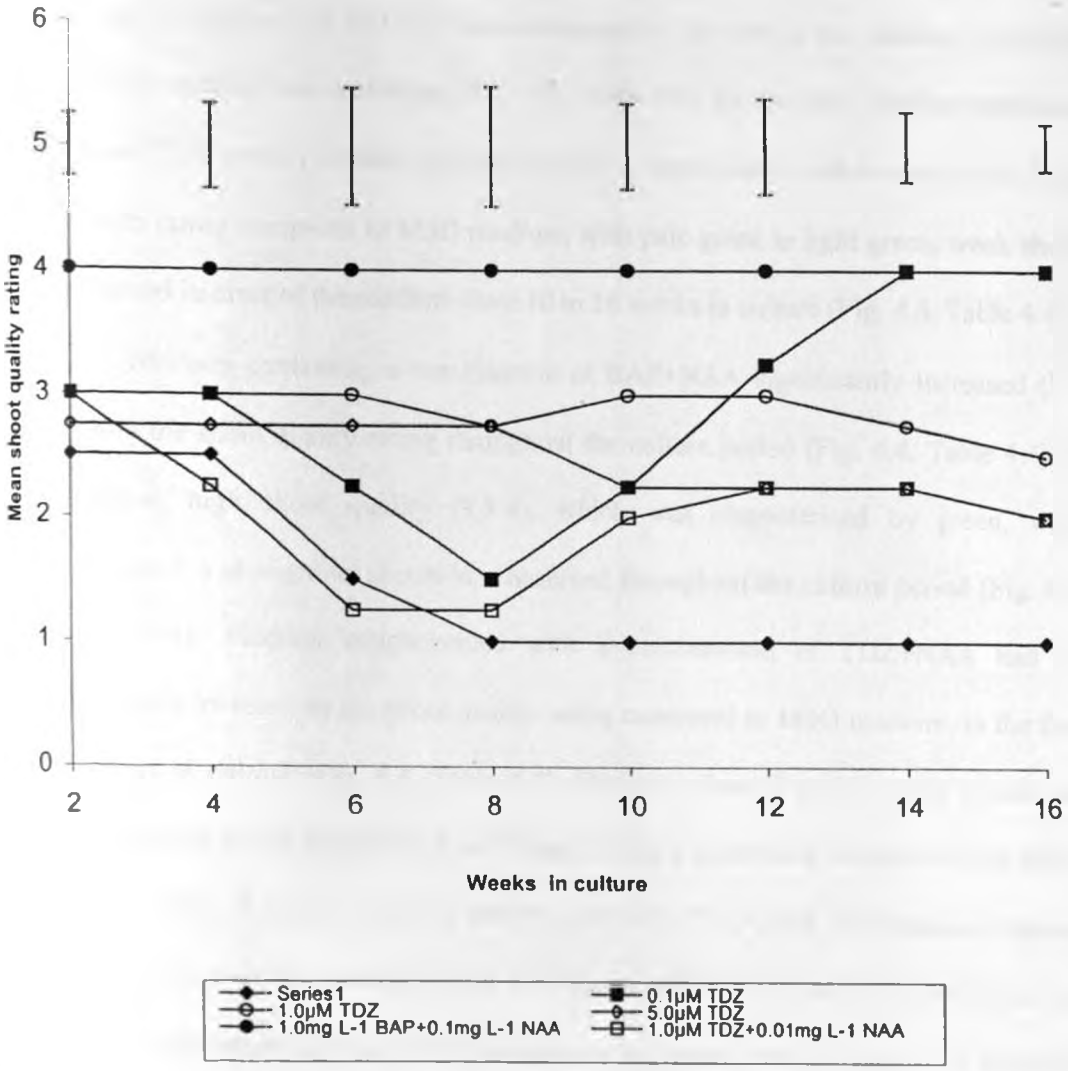
Table. 4.4 Effect of TDZ at various concentrations alone or in combination with NAA on the mean shoot quality rating[z] compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* cv. Rosita shoot-tip cultures

PGRs	Weeks in culture							
	2	4	6	8	10	12	14	16
MSO	2.5b[y]	2.5b	1.5de	1d	1e	1d	1d	1e
0.1µM TDZ	3b	3b	2.25cde	1.5cd	2.25cd	3.25ab	4a	4a
0.4µM TDZ	3b	2.75b	2.75bc	2.5bc	3.5ab	3.5ab	3b	3b
1.0µM TDZ	3b	3b	3abc	2.75b	3bc	3bc	2.75bc	2.5c
5.0µM TDZ	2.75b	2.75b	2.75bc	2.75b	2.25cd	2.25c	2.25c	2d
1.0mg L^{-1} BAP+0.01mg L^{-1} NAA	4a	4a	3.5ab	3.5ab	4a	4a	4a	4a
1.0mg L^{-1} BAP+0.1mg L^{-1} NAA	4a	4a	4a	4a	4a	4a	4a	4a
1.0µM TDZ+0.01mg L^{-1} NAA	3b	2.25b	1.25e	1.25d	2d	2.25c	2.25c	2d
1.0µM TDZ+0.1mg L^{-1} NAA	3b	3b	2.5bcd	2.5bc	3.25ab	3.25ab	3b	3b
Significance	***	***	***	***	***	***	***	***
W(Turkey's)	0.6	0.83	1.1	1.15	0.83	0.92	0.68	0.46
SE	0.25	0.34	0.47	0.48	0.34	0.38	0.28	0.19
CV	8.1	11.5	18.1	19.9	12.4	13.1	9.9	6.8

[z] = Scores were visual attributes rated on a 1-4 scale; 1 = brown, poor growth; 2 = pale green, severe chlorosis, fair to poor growth; 3 = light green, slight chlorosis, good growth; 4 = green, healthy and vigorous growth.

*** =significant at $P \leq 0.001$.

[y] = Mean separation using Turkey's. Values having the same letters within the same column are not significantly different at 5% level of probability



mg L-1= mg in a liter

Fig 4.4 Effect of TDZ at various concentrations alone or in combination with NAA on the mean shoot quality rating compared to a combination of BAP and NAA in *Alstroemeria aurantiaca* cv. Rosita shoot-tip cultures. Vertical bars represent standard error.

0.1, 0.4, and 1.0μM from 6 to 8 weeks in culture (Fig. 4.4, Table 4.4). However, in the following 12 to 16 weeks, there was a significant increase in the shoot quality rating over the control, in medium with lower TDZ levels (0.1 and 0.4) μM (Fig. 4.4, Table 4.4). Medium containing 0.1μM TDZ recorded the highest mean shoot quality rating (4), in the 14^{th} and 16^{th} week compared to the rest of the medium containing TDZ at various concentrations (Fig. 4.4, Table 4.4). In contrast, medium containing higher TDZ levels (1.0 and 5.0) μM recorded a significantly low increase in the shoot quality rating compared to MSO medium, with pale green to light green, weak shoots observed in most of the medium from 10 to 16 weeks in culture (Fig. 4.4, Table 4.4).

Medium containing a combination of BAP+NAA significantly increased ($P \leq 0.001$) the shoot quality rating throughout the culture period (Fig. 4.4, Table 4.4). In addition, high shoot quality (3.5-4), which was characterised by green, well-developed, and vigorous shoots was observed throughout the culture period (Fig. 4.4, Table 4.4). Medium supplemented with a combination of TDZ+NAA had no significant increase on the shoot quality rating compared to MSO medium, in the first 6 weeks in culture (Fig. 4.4, Table 4.4). However, from 8 to 16 weeks in culture, medium containing higher NAA at 0.1mg L^{-1} had a significant increase on the shoot quality rating (2.5, 3.23, 3.25, 3, and 3) at (8, 10, 12, 14, and 16) weeks in culture, respectively, over the control (Table 4.4). These results were similar to that observed in medium containing 0.4μM TDZ throughout the culture period (Table 4.4). Medium containing lower NAA at 0.01mg/l recorded a significantly low shoot quality rating (2, 2.25, 2.25, and 2) at (10, 12, 14, and 16) weeks in culture respectively (Fig. 4.4, Table 4.4). The low shoot quality rating was observed as pale green to light green, weak, shoots with poor growth. These results were comparable to those recorded in medium supplemented with 5.0μM TDZ, from 10 to 16 weeks in culture (Fig. 4.4,

Table 4.4). Significant differences were observed in the shoot quality rating between medium containing TDZ with low NAA (0.01mg L^{-1}) and high NAA (0.1mg L^{-1}) from 6 weeks in culture (Table 4.4). Better shoot quality was noticed in medium with higher NAA at 0.1mg L^{-1} (Table 4.4).

A combination of BAP+NAA significantly increased ($P \leq 0.001$) the quality of shoots throughout the culture period. In contrast, high shoot quality rating (3-4) was observed in medium containing TDZ at 0.1 to 0.4μM, only during the last 12 to 16 weeks in culture. In this regard, TDZ was only comparable to a combination of BAP and NAA at much lower concentrations (0.1 to 0.4μM TDZ), and during the last 12 to 16 weeks in culture.

Rooting of Alstroemeria aurantiaca cv Rosita shoots

Rooting was scored based on the number of cultures in a treatment initiating roots at the base of the shoot: - = no rooting; + = 1 out of four cultures rooted; ++ = 2 out of four cultures rooted; +++ = 3 out of four cultures rooted; ++++ = all four cultures rooted.

Shoots previously cultured in basal medium did not root, when transferred to rooting medium containing 3mg L^{-1} IBA (Table 4.5). Medium containing 0.4μM TDZ readily initiated rooting in all the cultures, when the shoots were sub-cultured in rooting medium for 4 weeks (Table 4.5, Plate 5). These results were similar to those observed in shoots previously cultured in medium supplemented with a combination of BAP and NAA (Table 4.5). Low rooting was observed in medium containing a higher TDZ level at 5.0μM TDZ (Table 4.5, Plate 5: Right). Cultures with multiple shoots rooted better (Plate 6).

Table 4.5 Effect of PGRs on subsequent rooting of *Alstroemeria aurantiaca* cv. Rosita shoots in medium containing 3mg L^{-1} IBA

PGRs in growth medium	3mg L^{-1} IBA (Rooting medium)
MSO	-
0.1μM TDZ	++
0.4μM TDZ	++++
1.0μM TDZ	++
5.0μM TDZ	+
1.0mg L^{-1} BAP+0.01mg L^{-1} NAA	++++

- = No rooting; + = 1 out of four cultures rooted; ++ = 2 out of four cultures rooted; +++ = 3 out of four cultures rooted; ++++ = all four cultures rooted.

Plate 5 *In- vitro* regeneration of plants from *Alstroemeria aurantiaca* cv. Rosita shoot-tip explants. Shoots rooted in medium supplemented with 3mg L^{-1} IBA after a subculture period of 4 weeks.

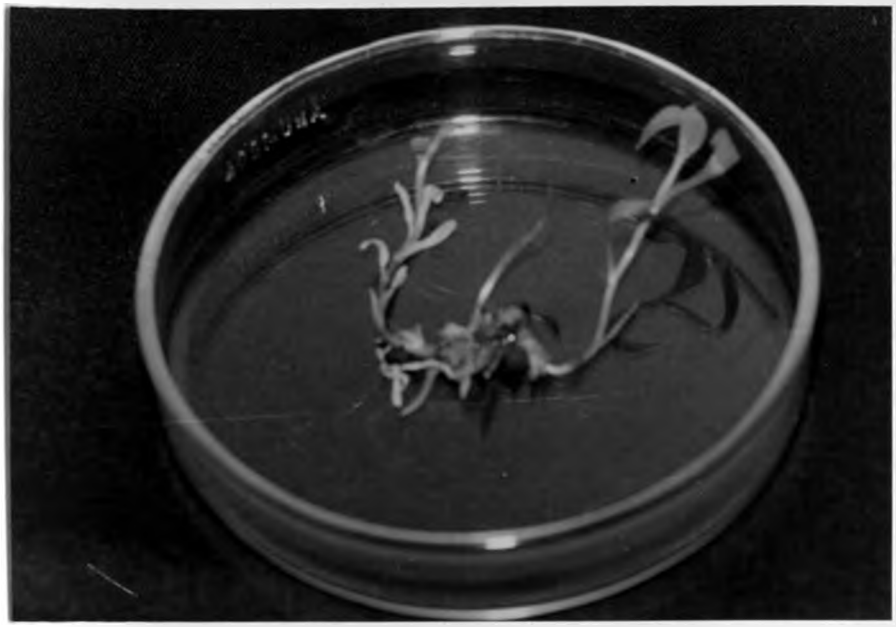


Plate 6 Rooted multiple shoots of *Alstroemeria aurantiaca* cv. Rosita in medium containing 3mg L^{-1} IBA after 4 weeks subculture period. Multiple shoots acclimatized easily compared to single shoots.

Discussions

As the number of shoots increased during the culture period, the number of leaves increased up to the 12th week, and stabilised in subsequent weeks for most treatments. Cultures that recorded high number of shoots in the 16th week had lower number of leaves per shoot, among treatments supplemented with TDZ. Shoot length declined with increase in number of shoots. High shoot quality rating was observed in cultures, which recorded high number of shoots.

Number of shoots

TDZ at 0.1, 0.4, and 1.0μM supported a steady increase in the number of shoots compared to that observed in medium containing a combination of BAP+NAA after 10 weeks in culture. Feijoo and Iglesias (1998), demonstrated the persistent response induced by TDZ in the in vitro multiplication of *Gentiana lutea* L. Aurantiaca Lainz. Suggesting a slow release of TDZ or because it stimulates the synthesis of endogenous cytokinins which may take time to cause a response (Kanakia and Demetrious, 1993). Further evidence is provided for the stimulation of endogenous cytokinins by TDZ due to the ability of cytokinin dependent bioassays achieving cytokinin autonomy (Capelle *et al.*, 1983). TDZ at 0.4μM had the highest number of shoots among TDZ treatments at 16 weeks in culture, and was comparable to that observed in medium containing a combination of BAP+NAA with lower concentration of NAA (0.01mg L^{-1}). Previously, TDZ has been reported to induce the regeneration of shoots with an efficiency comparable to or greater than that of other cytokinins (Kerns and Meyer, 1986; Fellman *et al.*, 1987; Fiola et al., 1990; Malik and Saxena, 1992a, 1992b). Recently, the possibility of TDZ substituting for a combination of auxin and cytokinin in inducing somatic embryogenesis in geranium hypocotyl cultures, was observed (Hutchinson *et al.*, 1996a; Visser *et al.*, 1992). In

the present study, the induction of shoots from shoot-tip explants, a response usually mediated by a combination of auxin and cytokinin, suggests that TDZ could have substituted for the auxin-cytokinin requirement for the regeneration of shoots in *Alstroemeria* cv. Rosita shoot-tip explants. Furthermore, the possibility of TDZ acting directly like a cytokinin in the induction of shoots can not be ruled out, as was reported by Mok *et al.* (1982). These findings indicate that TDZ could have acted directly as a cytokinin and or through the modulation of endogenous growth substances (auxins and cytokinins), to achieve a comparable response in the regeneration of shoots, to that obtained with a combination of BAP+NAA.

Increasing TDZ concentration to 5.0μM, supported shoot growth but did not promote shoot multiplication. TDZ has been reported to stimulate accumulation of endogenous growth substances (Hutchinson *et al.*, 1996a). Probably, at high levels of TDZ concentration, the level of endogenous hormones especially auxins and ethylene were increased, this together with the fact that the plants regenerated from apical shoot-tips suggests that the tissues were exhibiting apical dorminance (Bond and Alderson, 1993), and may have contained relatively high levels of auxins. TDZ at 5.0μM may have inhibited shoot proliferation in a similar manner.

A combination of BAP and NAA in the medium, recorded the highest number of shoots at lower NAA levels (0.01mg L^{-1}) up to 14 weeks in culture. Probably optimum conditions were provided by a combination of BAP and NAA for the multiplication of shoots from apical shoot-tip explants. However, medium containing higher levels of NAA at 0.1mg L^{-1} recorded a significantly lower increase in the number of shoots from the 6^{th} to 10^{th} week in culture. Auxins have been shown to enhance cell elongation compared to cell multiplication (Vanderhoef, 1980). These results support the evidence that NAA at higher levels (0.1mg L^{-1}) may have inhibited

shoot multiplication. However this inhibitory effect may have been overcome at 12 weeks in culture, when similar results were reported in medium containing either high or low NAA concentrations. Suggesting the possibility of hormonal interactions and or other plant mechanisms to overcome inhibitory effects of PGRs on growth. However medium containing higher levels of NAA recorded significantly lower results compared to those recorded in medium containing 0.4μM TDZ in the 16th week of culture.

Addition of NAA to medium containing TDZ at 1.0μM did not have a significant change on the number of shoots compared to a similar TDZ concentration without NAA. In fact, the number of shoots were significantly reduced in medium containing lower levels of NAA (0.01mg L^{-1}) in the 16th week in culture. Visser *et al.* (1992), demonstrated that inclusion of NAA to TDZ medium did not have an effect on somatic embryogenesis. Suggesting inhibition of shoot multiplication by auxins as reported by Salisbury and Ross (1991). Similarly, Murthy *et al.* (1998). reported inhibition of growth by TDZ in several plant systems. These results support the view that TDZ at relatively higher level (1.0μM) in combination with NAA, may have enhanced the accumulation of auxins and ethylene which might have inhibited the multiplication of shoots. Shoot multiplication in medium containing a combination of TDZ+NAA was generally lower compared to treatments containing TDZ at 0.1, and 0.4μM, and a combination of BAP+NAA. Conversely, Lin *et al.* (1997), reported high frequency in shoot regeneration from leaf explants in *Alstroemeria* L. in medium supplemented with a combination of 10μM TDZ and 0.5μM IBA. The number of shoots per explant (1.9 to 3.9) was comparable to that recorded in the present study using TDZ alone at 0.4μM (4.5), after 14 weeks of culture.

MSO medium supported the growth of single shoots from apical shoot-tip explants. These results suggest that axillary or adventitious shoot proliferation was not enhanced in MSO medium, supporting the hypothesis that PGRs influenced axillary and adventitious development of shoots from cutured explants (Murthy *et al.*, 1998; Skoog, 1980; Skoog and Schmitz, 1979). The presence of a combination of BAP and NAA was essential for the onset of shoot organogenesis from shoot-tip explants; when used alone, these compounds were found to be ineffective. These observations provide further evidence to the theory that morphogenic responses in plants are regulated by an intricate balance and interaction of phytohormones, namely auxins and cytokinins (Skoog and Miller, 1957; Trewavas, 1981).

A delay in response to TDZ at various concentrations compared to a combination of BAP+NAA was observed in *Alstroemeria* shoot-tip explants. Perhaps ,as a result of delayed TDZ activity, suggesting that optimum levels may not have been used, tissue sensitivity to TDZ could have been inhibited, time was required for TDZ to induce metabolic processes, which latter influenced multiple shoot regeneration and or inhibitory effects were experienced which were overcome after 8 weeks in culture. TDZ has been reported to cause inhibition of growth in several plant systems (Murthy *et al.*, 1998). Probably, TDZ causes inhibition of growth through the elevation of endogenous ethylene which has been reported to promote degradative processes, in addition to causing stem thickening and shortening (Beyer *et al.*, 1984; Esinger, 1983). Suggesting that in a similar fashion, TDZ may have inhibited the multiplication of shoots in the first 8 weeks in culture.

In conclusion, medium containing a combination of BAP+NAA recorded an increase in the number of shoots from 2 to 12 weeks in culture, after which there was no further increase. However, in medium containing TDZ at 0.1, 0.4, and 1.0μM,

although shoot multiplication was not achieved until after 8 weeks in culture, a sustained increase in the number of shoots was observed from 10 to 16 weeks in culture. A combination of BAP+NAA had a significant increase and highest number of shoots during the culture period. TDZ at 0.4μM recorded high number of shoots, which was comparable to that observed in medium containing BAP+NAA, with NAA at lower levels (0.01mg L^{-1}), in the 16th week in culture. Therefore, TDZ at 0.4μM can be used in the regeneration of shoots in *Alstroemeria* cv. Rosita shoot-tip cultures. Another added advantage of TDZ is it's persistent nature in tissues, which may lead to hormone autonomy, and achievement of a response for a relatively short duration of exposure (10 d), and at much lower concentration of TDZ used to achieve a similar response to a combination of BAP+NAA. Addition of NAA to TDZ had a significantly low increase in the number of shoots compared to 0.4μM TDZ, in the 14th to 16th weeks in culture. These results imply that TDZ could have substituted for the auxin and cytokinin requirement in the regeneration of shoots in *Alstroemeria* cv. Rosita shoot-tip cultures.

Shoot quality rating

Shoot quality rating is an important variable to consider in the in vitro propagation of plants (Ziv, 1991). This is because poor quality shoots, hardly survived subsequent transfers and did not withstand acclimatization as was reported in *Alstroemeria* by Ziv (1991).

Medium containing a combination of BAP+NAA recorded high quality of shoots throughout the culture period. Probably, a combination of BAP+NAA provided optimum conditions, which supported good shoot quality. An interaction of phytohormones namely auxins and cytokinins have been reported to enhance plant growth responses (Skoog and Miller, 1957; Trewavas, 1981), which may have

resulted in the high quality of shoots. These findings further suggest the possible involvement of NAA in the synthesis of chlorophyll.

TDZ at 0.1, 0.4, 1.0, and 5.0μM had no significant increase on the quality of shoots over the control, in the first 4 weeks in culture. Suggesting the possibility of other factors such as endogenous substances, nutrient salts in the medium (preece, 1995; Ziv, 1991), and light (Castillo *et al.*, 1997), in influencing the quality of shoots. In the subsequent 10 to 16 weeks, there was a significant increase in the quality of shoots in medium containing lower TDZ levels (0.1 and 0.4μM), over the control. Probably the nutrient salts in the control had no effect on the quality of shoots as a result of decreased endogenous growth substances. TDZ has been reported to protect the degradation of chlorophyll in detached barley leaves (You *et al.*, 1992). Visser *et al.* (1995), demonstrated that TDZ caused greening (increased chlorophyll content) in geranium cotyledons. Similarly, Greening of cotyledons treated with TDZ in intact peanut seedlings, suggesting the accumulation of chlorophyll content was demonstrated by Murthy et al. (1995). Furthermore, TDZ was reported to mimic cytokinin effects through the retention of chlorophyll (Mok et al., 1982). In addition, TDZ has been reported to cause the elevation of endogenous growth substances, including cytokinins (Hutchinson *et al.*, 1996a; Murthy *et al.*, 1998). Cytokinins have been reported to increase the synthesis of proteins some of which bind chlorophyll thereby increasing it's concentration in tissues, which may enhance the quality of shoots (Chebet, 1999; Mutui, 1999; Parthier, 1979; Lew and Tsuji, 1984). These results support the evidence that TDZ at (0.1 and 0.4μM) may have caused an increase in shoot quality directly by acting as a cytokinin and or indirectly through the elevation of endogenous cytokinins.

In contrast, medium containing higher levels of TDZ at 1.0 and 5.0μM TDZ recorded a significantly lower quality of shoots compared to medium containing TDZ at (0.1 and 0.4μM) and a combination of BAP+NAA in the last three weeks in culture. TDZ has been reported to induce the elevation of endogenous growth substances, including ethylene (Hutchinson *et al.*, 1997a). Ethylene in turn promotes degradative processes, which might have resulted in the poor quality of shoots (Beyer *et al.*, 1984; Chebet, 1999; Mutui, 1999). These results support the observations obtained in the present study, and suggest that TDZ at higher levels (1.0 and 5.0)μM could have lowered the quality of shoots as a result of TDZ- induced elevation of ethylene.

Addition of NAA to TDZ did not improve the quality of shoots, and increasing the NAA concentration to 0.1mg L^{-1}, only slightly increased the quality of shoots which was comparable to that observed in medium containing 0.4μM TDZ, but was lower than that observed in medium supplemented with a combination of BAP+NAA. Indicating that probably a balance in auxins and cytokinins were achieved at higher levels of NAA when in combination with TDZ. This is also supported by evidence that TDZ modulated endogenous growth substances and may have induced increased elevation of cytokinins (Hutchinson *et al.*, 1996a), which in the presence of NAA may have improved the quality of shoots. In this case, high auxin in the presence of high cytokinins may have provided better conditions, which enhanced the quality of shoots. However, the hormonal balance achieved with TDZ+NAA may have been lower than that attained with a combination of BAP+NAA, resulting in lower shoot quality. Probably due to the negative effects on shoot quality related to accumulation of ethylene, observed in TDZ- mediated responses (Hutchinson *et al.*, 1997a).

MSO medium recorded low shoot quality throughout the culture period. Indicating that good shoot quality is enhanced by the interaction between PGRs and nutrient salts in the medium (Preece, 1995; Ziv, 1991).

In conclusion, the best shoot quality was observed in medium containing a combination of BAP and NAA, throughout the culture period. TDZ at lower levels (0.1-0.4) μM supported good quality of shoots. In contrast, TDZ at higher levels, resulted in low quality of shoots from the 10^{th} to 16^{th} week in culture. A combination of TDZ+NAA, at higher levels of NAA (0.1mg L^{-1}), supported good quality of shoots, but lower NAA levels at 0.01mg L^{-1} recorded low quality of shoots. Lower levels of TDZ at 0.1-0.4 μM can be used to propagate *Alstroemeria* cv. Rosita from shoot-tip explants, without lowering the quality of shoots. Our study reports for the first time the effect of TDZ at various concentrations alone or in combination with NAA on the quality of shoots compared to a combination of BAP+NAA in *Alstroemeria* cv. Rosita shoot-tip cultures. The study further suggests the possibility of TDZ at lower levels, substituting for the auxin –cytokinin requirement, by acting directly or through the modulation of endogenous growth substances to support good shoot quality.

Shoot length

The number of shoots is important in the development of efficient protocols, however, many shoots tend to compete for nutrients and result in shorter, poor quality shoots, which hardly survive subsequent transfers. In addition, very short shoots were difficult to handle, had fewer leaves and did not root easily.

Inclusion of TDZ at 1.0μM recorded the longest shoots up to 10 weeks in culture compared to a combination of BAP+NAA, before declining. The decline in shoot elongation could be due to increased competition for nutrients as a result of the high number of shoots observed in medium containing 1.0μM TDZ compared to

5.0μM TDZ, and or TDZ mimicking cytokinins, which inhibit shoot elongation but enhance shoot multiplication (Bruce, 1965; Mok *et al.*, 1982). At 16 weeks in culture, medium containing 5.0μM TDZ recorded the highest shoot elongation compared to that recorded in the rest of the medium containing TDZ at various concentrations. TDZ has been reported to modulate endogenous levels of auxins (Hutchinson *et al.*, 1986a). These results support the evidence that elevated auxins may also be responsible for the increased shoot elongation. Furthermore, reduced competition for nutrients as a result of lack of multiple shoots can not be ruled out.

A combination of BAP+NAA in the medium, supported shoot elongation, compared to that recorded in medium with 5.0μM TDZ but shorter than 1.0μM TDZ. In the same medium, there was an increase in number of shoots, which later stabilised and shoot elongation improved. Previously, competition for nutrients to support different growth processes has been reported (Janicks, 1986).This is because in the first 10 weeks of culture, a combination of BAP+NAA recorded the highest number of shoots, which might have occurred at the expense of shoot elongation. Growth processes have been reported to be regulated by an intricate balance in phytohormones, namely auxinns and cytokinins (Skoog and Miller, 1957). Probably medium containing a combination of BAP+NAA at the given concentrations provided optimum conditions for shoot elongation once shoot multiplication stabilised. In addition, the ratio of TDZ: NAA used was more important than the concentration of NAA for shoot elongation.

A combination of TDZ+NAA significantly reduced shoot elongation compared to TDZ at (1.0 and 5.0μM), and BAP+NAA. Inhibitory responses to shoot elongation have been reported in auxin mediated responses, especially when supraoptimal levels are implied (Vanderhoef, 1980). TDZ could have induced the accumulation of auxins

(Hutchinson et al., 1997a), which in the presence of exogenously applied auxins, may have resulted in inhibitory effect on shoot elongation. In addition, whenever supra-optimal levels of auxins are achieved, ethylene accumulation can not be ignored (Suttle *et al*). The later growth regulator has been shown to inhibit shoot elongation (Beyer *et al.*, 1984). The differences in shoot elongation between medium supplemented with a combination of TDZ+NAA could not be systematically attributed to the difference in concentration of NAA used. Shoot length also decreased with increase in the number of shoots. This was probably due to the competition for nutrients and or effect of PGRs. This is further supported by the hypothesis that a high ratio of cytokinin: auxin promotes shoot multiplication but may not promote shoot elongation and a low ratio of cytokinin: auxin promotes shoot elongation. This is consistent with the suggestion on TDZ substituting for cytokinin and auxin requirement.

Basal medium caused a low increase in shoot elongation. Several authors have reported the importance of plant growth substances in influencing the growth and development of shoots (Janick, 1986; Salisbury and Ross, 1991; Hartman *et al.*, 1990). The low increase in shoot elongation observed in MSO medium, could be attributed to inadequate plant growth substances in the tissues, and as a result only the limited endogenous plant growth substances were used to support growth (Hill, 1980).

Low TDZ levels at (0.1 and 0.4μM) did not support satisfactory shoot elongation, indicating the importance of optimum hormonal levels for the growth and development of shoots (Janick, 1986). In addition, among TDZ treatments, more number of shoots were observed at (0.1 and 0.4μM TDZ) and probably shoot multiplication may have been enhanced compared to shoot elongation.

We can conclude that, TDZ at 1.0μM recorded the highest shoot elongation in the first 12 weeks in culture. TDZ at 5.0μM had the highest shoot elongation in the 14th and 16th week in culture. Lower levels of TDZ (0.1-0.4) μM, maintained low shoot elongation, perhaps as a result of competition from the high number of shoots, and or low response caused by low concentration of TDZ. A combination of BAP and NAA had a steady increase in shoot elongation throughout the culture period. These results were comparable to that observed in medium containing 5.0μM TDZ. Addition of NAA to TDZ at higher levels (0.1mg L^{-1}), had low increase in shoot elongation. On the other hand, NAA at lower levels (0.01mg/l), had no significant increase in shoot elongation except in the 16th week in culture.

Number of leaves

Growth, in a restricted sense, refers to an irreversible increase in size, reflecting a net increase in protoplasm, brought about by increases both in cell size and the number of cells. Growth can be measured by increases in plant or organ size. The number of shoots as a measure for growth has been reported. However, very few authors have looked at the number of leaves as a measure of growth under in vitro conditions. This variable was important in monocotyledonous plants used in the study, especially Tuberose and *Alstroemeria*. This is because, shoots that had fewer leaves, did not root readily. This may be attributed to the fact that the rooting process involves carbohydrates, PGRs and some cofactors, which are produced in the leaves (Janick, 1986).

TDZ at higher levels (1.0 and 5.0)μM, had a significant increase on the number of leaves relative to the control, throughout the culture period. Medium containing

1.0μM TDZ caused a general increase in the number of leaves from 2nd to 12th week in culture, after which a decline was observed up to the 16th week. However, TDZ at 1.0μM recorded a significantly higher increase in the number of leaves in relation to medium containing 5.0μM TDZ from 4 to 12 weeks in culture. Related studies point to the evidence that TDZ promoted shoot regeneration with a higher efficiency compared to other phytohormones (Murthy *et al.*, 1998). TDZ could also substitute for auxin and cytokinin requirement with greater efficiency in somatic embryo development in geranium hypocotyl explants (Hurtchinson *et al.*, 1996a; Visser *et al.*, 1992). TDZ mediated growth and development of leaves in *Alstroemeria* can be related to the above findings. Suggesting that TDZ at higher levels 5.0μM did not support as many leaves per shoot compared to low TDZ levels at 1.0μM. This could be due to the evidence provided by Janick (1986), who reported that PGRs had promotive and inhibitory effects on growth, and this was greatly influenced by the concentration in the tissues. Very low levels had little influence, while very high levels were inhibitory. Suggesting the requirement for an optimum level to achieve satisfactory growth. We can speculate that 5.0μM was too high and inhibited the growth of leaves more than medium containing 1.0μM TDZ from 4 to 12 weeks in culture. However at 14 to 16 weeks in culture, medium containing 5.0μM TDZ recorded a significantly high number of leaves, which was comparable to that observed in medium containing 1.0μM TDZ. This could be attributed to the reduced competition for nutrients (Janick, 1986; Salisbury and Ross, 1991), due to the single shoots maintained in medium containing 5.0μM TDZ compared to 1.0μM TDZ where more shoot regeneration was observed.

Medium containing TDZ at relatively low levels (0.1μM) recorded low number of leaves similar to that observed in medium containing 0.4μM, but lower than

medium containing high levels of TDZ at 1.0 and 5.0μM. Plant growth responses are regulated by an intricate balance in phytohormones (Skoog and miller, 1957). Besides, optimum levels and ratios have to be used to elicit appreciable responses. Very low hormonal levels in the tissues have been reported not to cause effective response in growth (Janick, 1986). Probably TDZ at lower levels (0.1 and 0.4)μM, did not provide optimum conditions for the development of leaves, suggesting that the level might have been too low to cause appreciable increase in the number of leaves.

Medium supplemented with a combination of BAP+NAA recorded an increase in number of leaves and was comparable to 1.0 and 5.0μM TDZ, in the last 14 and 16 weeks in culture. Possibly, pointing to the importance of the ratio of BAP: NAA used compared to the levels of the PGRs, in the growth and development of leaves. Possibly, a combination of BAP+NAA provided optimum conditions for the development of leaves, although it was lower than that observed in medium containing 1.0μM TDZ at 8 to 12 weeks in culture, and only recording a higher increase in the 16^{th} week. These findings could suggest that TDZ at 1.0μM could have substituted for auxin and cytokinin requirement for the growth of leaves with a higher efficiency from 8 to 12 weeks in culture, the efficiency, reducing as the number of shoots increased. These results could also be attributed to the antagonistic response observed between increased shoot proliferation and number of leaves per shoot. Probably due to increased competition for nutrients. However, 5.0μM TDZ supported the growth of leaves with a comparable efficiency to that achieved by a combination of BAP+NAA. Similar results were reported by Hutchinson *et al.* (1996a), in geranium hypocotyl tissues treated with TDZ to stimulate somatic embryogenesis compared to a combination of IAA and BAP.

A combination of TDZ+NAA in the culture medium, significantly reduced the number of leaves compared to 1.0μM TDZ. Slow release of TDZ in tissues has been reported in gentiana plants (Feijoo and Igllesias, 1998). This may contribute to the slow response observed in the growth of leaves in medium containing a combination of TDZ+NAA. The addition of NAA to TDZ in the culture medium, May have resulted in an accumulation of auxins and ethylene which could have inhibited the growth and development of leaves. Nevertheless, the involvement of auxins in the growth and development of leaves can not be ruled out. This is shown when the number of leaves is significantly reduced in medium containing low levels of NAA (0.01mg L^{-1}). The concentration of NAA used was more important than the ratio of TDZ: NAA, with lower levels of NAA (0.01mg l^{-1}), significantly reducing the number of leaves compared to a combination of BAP+NAA. Furthermore, a comparable number of leaves was observed in medium containing 0.1μM TDZ during the same culture period. This low increase in the number of leaves may be attributed to the lack of adequate levels of NAA which in turn could have resulted in a poor hormonal balance (Skoog and Miller, 1957), which was not conducive for the increased growth of leaves.

MSO medium recorded very few healthy, visible and well-developed leaves throughout the culture period. The growth and development of plant organs has been reported to be regulated by an intricate balance and interaction of phytohormones, especially auxins and cytokinins (Janick, 1986; Skoog and Miller, 1957; Trewavas, 1981). Leaves develop from buds which are centers of growth consisting of actively growing undifferentiated cells known as meristems. Buds can give rise to leaves, flowers or both leaves and flowers. Wang *et al.* (1986), reported the induction of bud break and development in dormant apple (*Malus domestica* Borkh.) trees after

application of TDZ. In this case, TDZ had a cytokinin like effect since cytokinins have been reported to induce bud break (Skoog and Schmitz, 1979). In addition, leaves develop from lateral buds and have been shown to be influenced by cytokinins (Salisbury and Ross, 1991).These results suggest that phytohormones and especially cytokinins are important for the growth and development of leaves. This could explain why MSO medium recorded very few healthy, visible, and well-developed leaves.

In conclusion, TDZ at 1.0μM recorded the highest number of leaves in the first 14 weeks in culture. At 16 weeks in culture, a more steady increase in shoot multiplication may have been favoured at the expense of leaf induction. A combination of BAP and NAA maintained a steady increase in the number of leaves throughout the culture period, and at 16 weeks, recorded the highest number of leaves. This is because a high ratio of a combination of cytokinins and auxins has been reported to support growth of stems, leaves and buds (Skoog, 1980). Furthermore, at 12 weeks of culture, shoot multiplication in medium with BAP+NAA had stabilised which could have allowed for the development of leaves.

Shoots recorded in medium containing 5.0μMTDZ also had a large number of leaves, probably as a result of reduced competition for nutrients and the longer shoot length achieved. TDZ at lower levels (0.1-0.4) μM, resulted in multiple shoots and on average, fewer number of leaves per shoot, may be as a result of competition for particular inductive stimuli and or nutrients. Medium supplemented with a combination of TDZ and NAA had a lower number of leaves compared to TDZ alone at 1.0μM.

Rooting

When shoots were transferred from medium containing TDZ to rooting medium supplemented with 3mg L^{-1} IBA (Gabryszewska, 1995) , roots appeared after one month in culture. Again, high TDZ levels (5.0μM) inhibited rooting. Huetteman and Preece (1993) and Lu (1993), reported inadequate rooting in TDZ-induced shoots, when supra-optimal levels were used.

CHAPTER FIVE

The potential of using thidiazuron in *in- vitro* propagation of *Ornithogalum saundersiae*

Abstract:

Direct adventitious shoot regeneration was achieved in shoot-tip explants of *Ornithogalum saundersiae* cultured on medium amended with various concentrations of thidiazuron (TDZ). TDZ at 5 μM induced shoot regeneration with a higher efficiency than that achieved with 0.5 mg L^{-1} BA and 2 mg L^{-1} NAA used in a previously published protocol (Nayak and Sen 1995). The plantlets previously cultured in medium amended with 5 μM TDZ successfully rooted and bulbed after transfer to basal medium.

Introduction

Many *Ornithogalum* species originated from South Africa and a few in the Mediterranean region. There are about 150 species with a few of economic importance. It is believed to have been introduced to kenya by the white settlers in the 70's (HCDA, 1996)

Ornithogalum Saundersiae belongs to the liliaceae family (Van Scheepen, 1991). Currently, the crop has gained much importance as an export crop in Kenya (HCDA, 1996). This is because of it's ease of growth even under out door conditions (Wabule, 1991). It is not also attacked by pests.and grows well under high light intensity. However it is affected by fungi, bacteria, and viruses (De hertogh and Le Nard, 1993).

Ornithogalum saundersiae is mainly propagated by bulbs. It is a herbaceous perennial and the bulbs are very prolific with a multiplication rate of 6-12 daughter bulbs from a single mother bulb. Seeds can be used, however, they take long to mature.

Ornithogalum saundersiae has become one of the most important cut flowers grown in Kenya (Mayo, 2000). This is because of the low level of management required by the crop. It can be grown successfully under outdoor conditions and is affected by few insects and pests. However, the expansion of this crop is threatened by fungal, bacterial and viral diseases (De hertogh and Le Nard, 1993; Wabule, 1991; Wangai and Bock, 1996).

Micropropagation has been used for the rapid multiplication of clean plants and genetic manipulations in a number of plant systems (Hartmann and Kester, 1985). A few studies have been conducted on the micropropagation of *Ornithogalum* spp. These have mainly included *Ornithogalum umbellatum* and *Ornithogalum thyrsoids* (De hertogh and Le Nard, 1993). A combination of BAP and NAA based on the Skoog and Miller theory (1957), was used in the regeneration medium, and bulb scales or shoot-tip explants cultured as initial explants. Nayak and Sen (1995), demonstrated a rapid and stable propagation of *Ornithogalum umbellatum* L. in long-term callus cultures using a combination of 0.5mg/l BA and 2mg/l NAA. TDZ has been reported to stimulate morphogenic responses with a comparable or higher level of activity compared to other adenine type cytokinins or a combination of auxins and cytokinins (Hutchinson *et al.*, 1996a; Murthy *et al.*, 1998). To our knowledge, there are no reports on TDZ-mediated shoot regeneration from *Ornithogalum saundersiae* shoot-tip cultures.

The overall objective of this study was

To determine the role of TDZ in morphogenesis and plant regeneration in *Ornithogalum saundersiae* shoot-tip cultures.

The specific objectives were:

1. To determine the effect of TDZ alone at various concentrations in stimulating morphogenic responses and regeneration of plantlets in *Ornithogalum saundersiae* shoot-tip cultures compared to a combination of BA and NAA.
2. To investigate the effect of TDZ alone at various concentrations compared to a combination of BA and NAA on subsequent rooting and bulbing in MSO medium

Materials and Methods

Site

Ornithogalum saundersiae stock plants with both shoots and bulbs were obtained from Mbugua farm in Limuru. Limuru is at an altitude of 1800-2100m. a.s.l. The area experiences a bimodal distribution of rainfall, with long rains in March-June and short rains in October-December. The annual rainfall is between 700-1000mm. The mean maximum day temperature is about 23°C and mean minimum of 15°C.

Plant Material

Ornithogalum saundersiae plants were grown under outdoor conditions with minimal supplemental irrigation during the dry seasons (January-March) and (July-September). Bulbs were dipped in benlate before planting at 5-10cm deep, on 1m wide raised beds. During land preparation, organic manure from poultry droppings and wood shavings was incorporated with DAP at 125kg/ha. The bulbs were planted at a spacing of 20cm between rows and 20cm within rows depending on the soil

status, making 4 rows per 1m wide bed. Manual weeding was done to keep the beds weed free. The crop was sprayed against fungal infections (stem rot, botrytis e.t.c.).and spider mites using Dithane M45 and Rogor E, respectively. Top dressing with CAN was done at 100 kg N/ha in a split application at 30 and 60 days after planting.

Preparation of explant and sterilisation

Ornithogalum saundersiae bulbs were obtained from three months old crop. The bulbs were cleaned with detergent and rinsed in running tap water for 15 minutes. Excised tips (1-2cm) long were placed in tap water in a beaker. The water was drained and the tips were immersed for 5 minutes in 95% alcohol. The alcohol was drained and the tips were rinsed in sterile distilled water for 3 minutes. The tips were placed in 0.5% NaOCl containing 'Tween 20' (2 drops/ 100mL solution), for 20 minutes, washed in three changes of distilled water and placed in a dry sterile petri dish before, shoot-tips (0.5-1.0mm long), consisting of an apical dome and one to two leaf primodia were excised under a dissecting microscope and inoculated.

Culture of shoot-tip explants

Each explant was cultured per universal bottle containing 10mL of medium. Four explants were prepared per treatment. The medium consisted of MS (Murashige and Skoog, 1962) salts, B5 (Gamborg *et al.*, 1968) vitamins, 30 g/l sucrose, and PGRs. The combination and concentration of BA and NAA used in this study were found optimum for shoot regeneration in *Ornithogalum spp* (Nayak and Sen, 1995). The level of BA and NAA used by Nayak and Sen (1995) had better results than other levels used during preliminary studies. As a result, they were included in the PGR treatments.

A total of 8 treatments, each replicated four times and arranged in a completely randomised design (CRD) were used as follows:

1. MSO
2. 0.1μM TDZ
3. 0.4μM TDZ
4. 1.0μM TDZ
5. 5.0μM TDZ
6. 2mg L^{-1} NAA
7. 0.5mg L^{-1} BAP
8. 0.5mg L^{-1} BAP+2mg L^{-1} NAA*

*= Protocol according to (Nayak and Sen, 1995)

The explants were maintained on the medium for four weeks after which they were sub-cultured after every month to the same medium except shoots previously cultured in medium containing TDZ alone. Shoots from the later media, were exposed to TDZ treatments for 10 days before they were transferred to MSO medium. The 10 d exposure was deduced from preliminary experiments based on the survival of shoot-tip explants. The pH of all media was adjusted to 5.7 before autoclaving at 1.4 kg cm^{-1} for 20 min. The cultures were placed on shelves set at 25± 2°C and illuminated (16-h photoperiod; 70-78umol $m^{-2}s^{-1}$) by cool-white fluorescent tubes. Data for mean number of shoots were recorded after 4 and 10 weeks in culture.

Statistical analysis

The treatments consisted of four replications and the experiment was repeated twice. Data on Percent rooting and bulbing were subjected to arc sine ($\sqrt{x}/ 100$) transformation prior to statistical analysis. Data were analysed using analysis of variance (GENSTAT statistical software (Lane and Payne, 1996), and the means were compared by Turkey's procedure at 5% level of probability.

Results

Number of shoots

The PGRs had a significant effect ($P \leq 0.001$) on the number of shoots at 4 and 10 weeks in culture (Table 5.1). TDZ at various concentrations induced direct adventitious shoot proliferation from *Ornithogalum saundersiae* shoot-tip cultures (Plate 7). The number of shoots increased with increasing concentrations of TDZ during the culture period. Most of the shoot-tips cultured in MSO medium only slightly elongated, and were of poor quality. In addition, there was no proliferation of shoots from shoot-tip explants in medium without any PGRs (Table 5.1). Medium supplemented with 2.0mg L^{-1} NAA alone, did not support any shoot growth, and the shoot-tip explants turned brown and died after 7d in culture (Table 5.1). However, BAP alone at 0.5mg L^{-1} had a significant increase on the number of shoots (2.5 and 6.75) at 4 and 10 weeks in culture, respectively (Table 5.1).

A combination of 0.5mg L^{-1} BA and 2.0mg L^{-1} NAA caused a highly significant increase in the number of shoots (7.0 and 22.5) at (4 and 10) weeks in culture, respectively (Table 5.1). These results were comparable to those observed in medium containing TDZ at (0.1 and 0.4) at 4 weeks in culture, and (0.1, 0.4, and 1.0μM TDZ) at 10 weeks in culture (Table 5.1). Medium containing TDZ at 5.0μM recorded significant ($P \leq 0.001$) and highest number of shoots (15.25 and 34.25) at (4 and 10) weeks in culture respectively (Table 5.1). The number of shoots observed in medium containing 5.0μM TDZ at 10 weeks in culture, were comparable to those recorded in medium containing 1.0μM TDZ (27.75) (Table 5.1).

Table 5.1 Effect of TDZ alone at various concentrations on the mean number of shoots compared to a combination of BA and NAA in *Ornithogalum saundersiae* shoot-tip cultures

PGRs	4-week cultures	10-week cultures
	Number of regenerating shoots per explant	Number of regenerating shoots per explant
MSO	1e	1c
0.1μM TDZ	6.0d	21.75b
0.4μM TDZ	9.5bc	25.0b
1.0μM TDZ	10.75b	27.75ab
5.0μM TDZ	15.25a	34.25a
2.0mg L^{-1} NAA	0.0e	0.0c
0.5mg L^{-1} BA	2.5e	6.75c
0.5mg L^{-1} BA+2.0mg L^{-1} NAA	7.0cd	22.5b
Significance	***	***
SE	1.315	3.6
W (Turkey's)	3.1	8.4
CV	20.2	20.8

*** Significant at $P \leq 0.001$ level of probability

Rooting and bulbing

When the shoots were transferred to MSO medium, rooting and bulbing occurred in shoots previously cultured in medium containing 0.1-5.0μM TDZ (Plate 8, Table 5.2). However, much lower bulbing was noticed in medium containing BA alone and a combination of BA and NAA compared to TDZ at (1.0 and 5.0μM) (Table 5.2).

Table 5.2 Effect of TDZ alone at various concentrations compared to a combination of BA and NAA on subsequent bulbing and rooting of Ornithogalum saundersiae shoots after subculturing for six weeks in MSO medium.

PGRs	Percent shoots rooting[z]	Percent shoots bulbing[z]
MSO	0d[y]	0d
0.1μM TDZ	71.3b	42.5bc
0.4μM TDZ	70.9b	47.7bc
1.0μM TDZ	85.8ab	75.8a
5.0μM TDZ	100a	86a
2.0mg L^{-1} NAA	0d	0d
0.5mg L^{-1} BA	33.8c	33.8c
0.5mg L^{-1} BA+2.0mg L^{-1} NAA	95.9a	55.9b
Significance	***	***
SE	0.139	0.086
W (Turkey's)	0.327	0.201
CV	19.2	18.3

*** = significant at $P \leq 0.001$ level of probability

[z] = Actual data are presented, but data were transformed, based on arcsin ($\sqrt{x}/100$) transformed values for analysis.

[y] = Mean separation within columns by Turkey's procedure. Values followed by the same letter are not significantly different at 5% level of probability.

Plate 7 Induction of direct adventitious shoot proliferation from *Ornithogalum saundersiae* shoot-tip explant exposed for 10 d in medium containing 5.0μM TDZ at 10 weeks in culture after transfer to MSO medium.

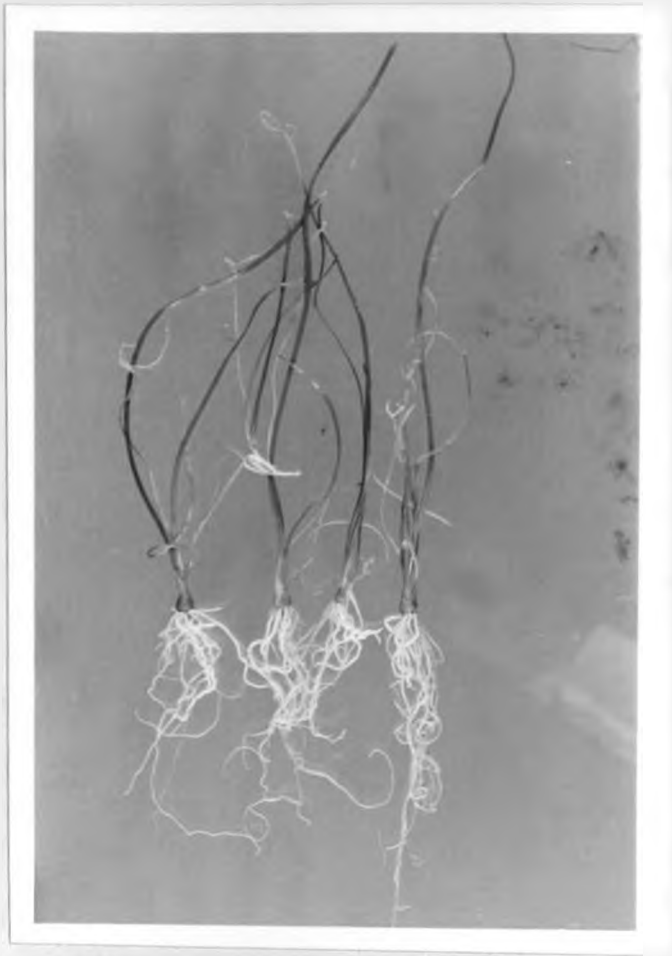


Plate 8 Rooting and Bulblet formation of *Ornithogalum saundersiae* shoots cultured in 1.0μM TDZ and transferred to MSO medium at 4 weeks subculture.

Discussions

TDZ induced direct adventitious shoot regeneration from *Ornithogalum saundersiae* shoot-tip explants without intermediate callus formation. Muthy *et al.* (1998), reviewed similar findings in previous studies involving TDZ-mediated shoot regeneration in a number of plant systems, including woody species. Likewise, the present studies with *Alstroemeria* and carnations demonstrated similar findings. TDZ at (1.0-5.0μM) may have acted directly as a cytokinin (Mok *et al.*, 1982), or through the modulation of endogenous growth substances (Capelle et al., 1983; Hutchinson *et al.*, 1996a), with higher efficiency compared to a combination of BA and NAA. We could also suggest that TDZ may have substituted for the auxin and cytokinin requirement for regeneration of shoots as was previously demonstrated in the development of somatic embryos in geranium tissue cultures (Hutchinson *et al.*, 1996a; Murthy *et al.*, 1996a; Visser *et al.*, 1992).

TDZ at 1.0 and 5.0μM had the highest rooting and bulbing of shoots after sub-culturing in MSO medium for 14 weeks. Probably optimum level of auxin and cytokinin was achieved at (1.0 and 5.0μM TDZ), resulting in high rooting and bulbing of shoots in subsequent MSO medium, relative to medium supplemented with a combination of BA+NAA.

In summary, TDZ at 1.0-5.0μM induced the highest number of shoots from *Ornithogalum saundersiae* shoot-tip explants compared to a combination of BA and NAA as previously used in a published protocol (Nayak and Sen, 1995). Furthermore, the shoots readily rooted and formed bulbs when transferred to MSO medium.

CHAPTER SIX

The potential of using thidiazuron in *in-vitro* propagation of *Dianthus caryophillus* L. cv. Yair

Abstract:

The potential of thidiazuron (TDZ) in stimulating morphogenetic responses and plant regeneration from carnation (*Dyanthus caryophillus* cv. Yair) shoot-tip explants compared to the conventionally used combination of KIN and NAA was investigated. TDZ at 1 to 5 μM promoted shoot regeneration with an efficiency greater than that obtained by conventionally used combination of 0.2 mg L^{-1} KIN and 0.2 mg L^{-1} NAA. Shoot-tips cultured for only 3 days on TDZ- supplemented medium before transfer to basal medium was sufficient for inducing shoot regeneration.

Introduction

The carnation (Caryophyllaceae; *Dianthus caryophyllus*) has been cultivated for over 2000 years. About 300 B.C. Theophrastus wrote about "Dianthus," which translated from Greek means "Divine Flower," because of it's delightful fragrance. The species name, *caryophyllus*, was once used as a generic name for clove, the basic fragrance of the carnation (Besemer, 1980). The common name, carnation, is likely derived from "coronation," as the Greeks wove Dianthus flowers into crowns for their athletes.

The carnation is indigenous to the Mediterranean area. The native species bloomed only in spring as a reaction to increased photoperiods and temperature. Improvement of the native Dianthus began in the 16th century. The perpetual flowering race of carnation, leading to the American types, was developed in France

in 1840 and introduced into America in 1852. Since then, hundreds of cultivars have been developed for commercial flower production. The cultivar William sim produced in 1938-1939 by William Sim of North Berwick, Maine, was the greatest contribution to the present carnation industry. From one red flowering plant there have been mutations to White, Pink, Orange, and several variegated forms. Today, the Sim carnation strains are grown throughout the world.

Modern carnations have little resemblance to their ancestors for now they flower year-round, have long stems, much larger and fuller flowers, and a greater array of colours (Besemer,1980). The carnation is thought to have been introduced in Kenya by the white settlers in the early sixties (HCDA, 1996).

Blake (1962) described the typical flower as consisting of two pairs of bracts closely and pressed to a five-membered calyx which is formed into a tube with five terminal teeth. An average flower may contain 60 petals (± 20) and up to 30 stamens, the latter often reduced to rudimentary filaments. The superior ovary consists of two or three carpels and the fruit is a capsule.

Each stem forms a terminal flower and the inflorescence is a loose cyme. The flowering shoot can be marketed in one of two forms. Either the flower buds formed on short lateral shoots arising from the axils of the upper leaves are removed to leave one large, terminal flower on a long leafy stem ('standard' type), or the terminal flower bud is removed at an early stage to encourage more even development of the lateral flowers which then produce multiple flowered stem ('spray' or 'miniature' type) (Blake, 1962).

Carnation (*Dianthus caryophillus* L.) is one of the major floriculture crops initially grown in Kenya, and ranks second to roses (HCDA, 1998). Carnation cv. Yair is widely grown in Kenya by both small to medium scale farmers due to their

ease of management and the ability to grow under out door conditions. In addition, the carnation cv. Yair is white in colour and can be dyed to suit consumer preferences, thereby resulting to high demand. Carnations have been propagated vegetatively in most farms, using cuttings from previous season's crop (Besemer, 1980; Yako, 1999). This traditional method is both slow and increases the chances of infecting the subsequent crop, leading to low yield and poor quality flowers (Wabule *et al.*, 1991). This is because Carnations are highly infected by both viral and fungal diseases (Besemer, 1980; Muthome, 1994; Spamaaij, *et al.*, 1991). Besides, tissue culture and modern biotechnological techniques can be used as alternative and complementary tools to the classical means for carnation improvement, and availabillity of adequate clean materials. However, their application requires the development of a routine and effective regeneration procedure (Kitto, 1997; Yantcheva *et al.*, 1998).

Efficient protocols can be developed through manipulation of culture environment, nutrient salts in the medium, regeneration pathways, type of explant, and use of potent PGRs. The use of potent PGRs has been shown to be a simple, cheap, and faster method, which is also suitable for small laboratories in developing countries. Recently, *in-vitro* organogenesis in carnation shoot-tip explants with considerable progress using a combination of auxin and cytokinin based on the Skoog and Miller theory (1957), has been reported (Goerge and sherrington, 1984; Kallaak *et al.*, 1997; Mujib and Pal, 1994; Zimmerman *et al.*, 1986). Frey *et al.* (1992) and Sankhla *et al.* (1995), reported protocols for indirect somatic embryo formation. In addition, Yantcheva *et al.* (1998) established conditions for efficient direct somatic embryogenesis in selected carnation cultivars.

Initially, TDZ, a substituted phenylurea (N-phenyl-N'-1,2,3-thidiazol-5-ylurea; Dropp; TDZ), was primarily used as a cotton defoliant (Arndt *et al.*, 1976). In current

studies, TDZ in relatively small amounts (micromolar), has been shown to induce a variety of morphogenic responses with a high degree of efficiency (Fiola *et al.*, 1990; Lu, 1993; Malik and Saxena, 1992a; Saxena *et al.*, 1992).

Thidiazuron has also been used to induce somatic embryogenesis with a higher efficiency compared to a combination of auxin and cytokinin from cotyledons of white ash (Preece and Bates, 1990), tobacco leaf disc cultures (Gill and Saxena, 1992), geranium hypocotyl cultures (Hutchinson *et al.*, 1996a; Visser *et al.*, 1992), and peanut seedling cultures (Gill et al., 1993).

Despite it's high efficacy in inducing morphogenic responses in several plant systems, there are limited reports where TDZ has been used in the regeneration of carnation. Watad *et al.* 1997 used TDZ in the regeneration of shoots in Carnation cv White Sim under 3 different culture procedures: agar-gelled medium, liquid shaken medium, and an interfacial membrane raft floating on liquid medium. On agar-gelled medium, the number of regenerated shoots per explant from the first internode increased with increasing TDZ concentration (up to 18 μM). The number of regenerated shoots peaked at 4.5μM TDZ and 1.1μM TDZ in the other two cultures, respectively. Maximum regeneration on the raft was twice and three times that obtained with agar-gelled and liquid shaken media, respectively.

Zuker *et al.* (1995), reported the transformation of carnation plants produced by microprojectile bombardment, using a two step regeneration procedure based on the use of two different cytokinins, benzyladenine and thidiazuron for the production of adventitious shoots from stem segments of carnation cv. White Sim. Higher regeneration of shoots in medium containing TDZ compared to that which had KIN, BAP, and 2iP in carnation cultures was also reported by Nakano *et al.* (1994).

In all the above reports, no information is provided on TDZ-mediated regeneration of plants from shoot tip explants, and the optimum level of concentration and duration of exposure to TDZ of Carnation cv. Yair.

The main objective of this study was to investigate the role of TDZ in morphogenic responses and plant regeneration in carnation cv. Yair shoot-tip cultures.

The specific objectives of this study were:

1.To determine the effect of TDZ alone or in combination with NAA on regeneration of plantlets compaired to a combination of KIN+NAA from Carnation cv Yair shoot-tip cultures.

2. To asses the effect of dose and duration of exposure to TDZ on the regeneration of plantlets in Carnation cv. Yair shoot-tip cultures.

Materials and Methods

Site

Carnation cv. Yair stock plants were obtained from Mrs Margaret Wamboi's farm in Limuru. Limuru is at an altitude of 1800-2100m. a.s.l. The area experiences a bimodal distribution of rainfall, with long rains in March-June and short rains in October-December. The annual rainfall is between 700-1000mm. The mean maximum day temperature is about 23°C and mean minimum of 15°C.

Plant Material

The carnations were grown under outdoor conditions as spray type carnations, with minimal supplemental irrigation during the dry seasons (January-March) and (July-September). Carnation cuttings were planted on 1m wide raised beds. During land preparation, organic manure from well-rotted cow dung was incorporated with DAP at 50g/m^2. Furadan at 15-20g/m^2 was also added to the soil at planting to control pests, especially cutworms. Cuttings were planted at a spacing of 25cm between rows

experiments. The treatment with the best results based on the survival (greening) of shoot-tip explants was included in the study.

In the experiment involving the establishment of plants, 11 treatments, replicated four times and arranged in a CRD were used as follows:

1. MSO
2. 0.1μM TDZ
3. 0.4μM TDZ
4. 1.0μM TDZ
5. 5.0μM TDZ
6. 0.1μM TDZ +0.01mg L^{-1} NAA
7. 1 μM TDZ+0.01mg L^{-1} NAA
8. 1 μM TDZ+0.1mg L^{-1} NAA
9. 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA*
10. 0.2mg L^{-1} KIN
11. 0.2mg L^{-1} NAA

* based on protocols by (George and Sherrington, 1984; Zimmerman *et al.*, 1986)

Finally, the medium for the second experiment on the effect of dose and duration of exposure to TDZ was prepared as follows, MS salts, vitamins, 30gL^{-1} sucrose and (0.1, 0.4, 1.0, 5.0μM TDZ). The cultures were then exposed for (3, 10, 24, and 40 d) to TDZ.

A total of 16 treatments, replicated three times in a CRD were used as follows.

Dose	Duration of exposure (days)			
	3	10	24	>40
0.1μM TDZ	1	2	3	4
0.4μM TDZ	5	6	7	8
1.0μM TDZ	9	10	11	12
5.0μM TDZ	13	14	15	16

The pH of the media was adjusted to 5.0±0.1 before autoclaving at 1.4 kg cm^{-2} for 20 min. The cultures were placed on shelves set at 25°±2.0°C and illuminated (16-h photoperiod; 70-78 μmol m-2s-1) by cool-white fluorescent tubes.

The mean number of shoots, mean shoot length, mean quality rating, mean number of leaves and mean vitrification rating, were measured after (14, 21, 28, and 35 d). Shoot quality was rated based on visual attributes according to a scale we developed during the study. 1 = brown, poor growth; 2 = pale green, severe chlorosis, fair to poor growth; 3 = light green, slight chlorosis, good growth; 4 = green, healthy and vigorous growth. Vitrification is a physiological disdorder observed during micropropagation of plants. It is characterised by hyperhydricity of shoot tissues, which appear watery, succulent, and are difficult to handle during subsequent transfers. This condition was scored using a visual estimate based on a scale of 1 to 4: 1=no vitrification (normal appearance), 2= low vitrification (<50%), 3= high vitrification (>50%), 4= all shoots vitrified.

Statistical analysis

In the first experiment on establishment of carnation, the treatments consisted of four replications. The second experiment on the effect of dose and duration of exposure to TDZ on the regeneration of plants from carnation cv. Yair shoot-tip cultures, consisted of three replications. All the experiments were repeated at least twice. Data were analysed using analysis of variance (GENSTAT statistical software (Lane and Payne, 1996), and the means were compared by Turkey's procedure at 5% level of probability. Regression analysis was performed on data for the second experiment on the effect of dose and duration of exposure to TDZ on the regeneration of plantlets in carnation shoot-tip cultures.

Results

Number of shoots per explant

Generally, there was an increase in the number of shoots in all the treatments, except MSO medium, throughout the culture period (Fig. 6.1). During the first week in culture, the different treatments did not have a significant effect on the number of shoots (Table 6.1). In the second, third, and fourth weeks in culture, the treatments had a significant effect on the number of shoots (Table 6.1).

Medium supplemented with 0.2mg L^{-1} KIN or 0.2mg L^{-1} NAA alone, did not support the growth of shoot-tip explants, which turned brown and died after 5-7 days in culture. Consequently, data was not included in the results.

Basal medium recorded very low (1) number of healthy, visible, green shoots during the culture period (Fig. 6.1, Table 6.1). Single, healthy, and visible shoots were recorded in all treatments during the first week in culture (Fig. 6.1, Table 6.1).

TDZ induced multiple regeneration of shoots from carnation shoot-tip explants (Plate. 10: left). The regeneration of shoots increased with increasing level of TDZ (Table. 6.1, Fig. 6.1). TDZ at 0.1, 0.4, 1.0, and 5.0μM significantly increased ($P \leq 0.001$) the number of shoots from the 2nd to 4th week in culture (Fig. 6.1, Table 6.1). However, medium containing TDZ at 5.0μM recorded a significant ($P \leq 0.001$), and highest increase in the number of shoots (8.5, 20.25, and 32.25) at (2, 3, and 4) weeks in culture, respectively (Fig. 6.1, Table 6.1). In addition, friable to compact, pink to green callus was observed in medium containing TDZ at 5.0μM (Plate. 9).

A combination of TDZ+NAA at 1.0μM TDZ+0.01mg L^{-1} NAA and 1.0μM TDZ+0.1mg L^{-1} NAA had no significant increase on the number of shoots in the 2nd ,3rd , and 4th week in culture (Table 6.1). Medium containing 0.1μM TDZ+0.01mg

Table. 6.1 Effect of TDZ at various concentrations alone or in combination with NAA on the mean number of shoots per explant[z] in carnation cv. Yair compared to a combination of KIN and NAA.

PGRs	weeks in culture			
	1	2	3	4
MSO .	1[y]	1d	1f	1e
0.1μM TDZ	1	3.25c	8cd	13c
0.4μM TDZ	1	3.25c	8.75c	15c
1.0μM TDZ	1	5.75b	14b	24.75b
5.0μM TDZ	1	8.5a	20.25a	32.25a
0.1μM TDZ+ 0.01mg L^{-1} NAA	1	1.25d	3.75e	7d
1.0μM TDZ+0.01mg L^{-1} NAA	1	1.5d	2.5ef	3.75de
1.0μM TDZ+0.1mg L^{-1} NAA	1	1.75cd	2.5ef	4.75de
0.2mg L^{-1} KIN+0.2mg L^{-1} NAA	1	3.25c	6.75d	14c
Significance	n.s	***	***	***
SE	0	0.68	1.139	2.317
CV	0	20.8	15.2	18.1
W(Turkey's)	0	1.62	1.13	2.3

n.s, ***, = nonsignificant, significant at 0.001 respectively.
[y] = Mean separation using Turkey's procedure. Values followed by the same letter in the same column are not significantly different at 5% level of probability.
[z]=Healthy, green, visible, and well developed shoots counted

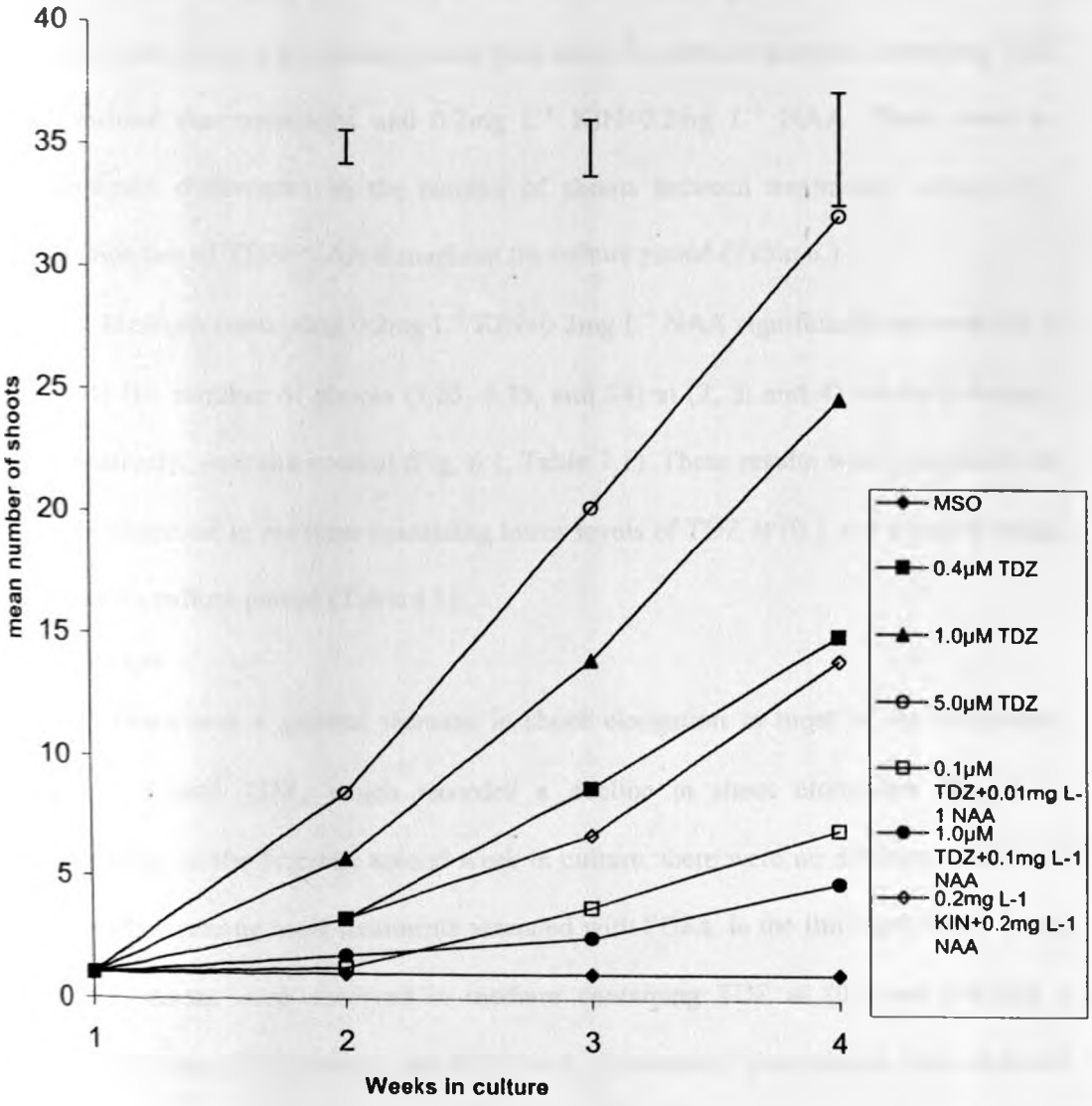


Fig.6.1 Effect of TDZ at various concentrations alone or in combination with NAA on the mean number of shoots compared to a combination of KIN and NAA in carnation shoot-tip cultures. Vertical bars represent standard error

L^{-1} NAA had no significant increase on the number of shoots in the 2nd week, however, in the 3rd and 4th week, a significant increase in the number of shoots (3.75 and 7) respectively, relative to the control was noticed (Fig. 6.1, Table 6.1). However, these results were significantly lower than those recorded in medium containing TDZ at various concentrations and 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA. There were no significant differences in the number of shoots between treatments containing a combination of TDZ+NAA throughout the culture period (Table 6.1).

Medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA significantly increased ($P \leq 0.001$) the number of shoots (3.25, 6.75, and 14) at (2, 3, and 4) weeks in culture, respectively, over the control (Fig. 6.1, Table 7.1). These results were comparable to those observed in medium containing lower levels of TDZ at (0.1 and 0.4μM) during the same culture period (Table 6.1).

Shoot length

There was a general increase in shoot elongation in most of the treatments, except 5.0μM TDZ, which recorded a decline in shoot elongation (Fig. 6.2). Generally, in the first and second week in culture, there were no differences in shoot elongation among most treatments amended with PGRs. In the third and fourth week, longer shoots were observed in medium containing TDZ at (0.1 and 0.4μM), a combination of TDZ+NAA, and KIN+NAA. Conversely, short shoots were observed in medium containing TDZ at (1.0 and 5.0μM), during the same culture period. The treatments (PGRs) had a significant effect ($P \leq 0.001$) on shoot elongation throughout the four weeks in culture (Table 6.2).

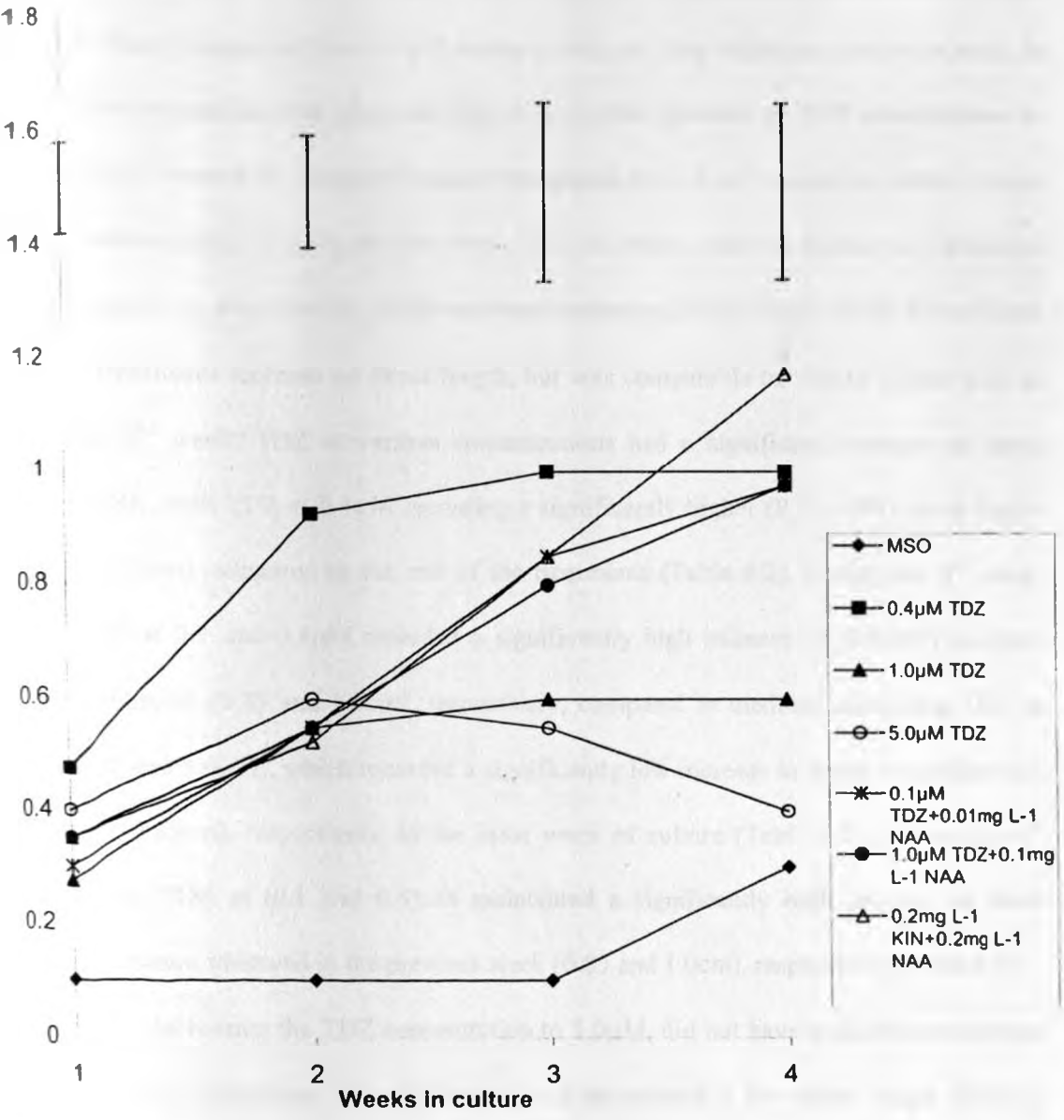
Shoot tip explants cultured in MSO medium did not show any shoot elongation (0.1, 0.1, and 0.1) at (1, 2, and 3) weeks in culture, respectively (Fig. 6.2, Table 6.2).

Table. 6.2 Effect of TDZ at various concentrations alone or in combination with NAA on the mean shoot length (cm) in carnation cv. Yair compared to a combination of KIN and NAA.

PGRs	weeks in culture			
	1	2	3	4
MSO	0.1d[y]	0.1c	0.1d	0.3c
0.1μM TDZ	0.55a	0.55b	0.85ab	0.85ab
0.4μM TDZ	0.475ab	0.925a	1a	1a
1.0μM TDZ	0.275cd	0.55b	0.6b	0.6bc
5.0μM TDZ	0.4abc	0.6b	0.55c	0.4c
0.1μM TDZ+ 0.01mg L^{-1} NAA	0.3bc	0.55b	0.85ab	0.975a
1.0μM TDZ+0.01mg L^{-1} NAA	0.45abc	0.55b	1.1a	1.125a
1.0μM TDZ+0.1mg L^{-1} NAA	0.35bc	0.55b	0.8ab	0.975a
0.2mg L^{-1} KIN+0.2mg L^{-1} NAA	0.35bc	0.525b	0.85ab	1.175a
Significance	***	***	***	***
SE	0.0805	0.0991	0.1596	0.1563
cv	22.3	18.2	21.2	19
W(Turkey's)	0.19	0.23	0.31	0.37

*** = significant at P<0.001.

[y] = Mean separation using Turkey's procedure. Values followed by the same letter in the same column are not significantly different at 5% level of probability.



g.6.2 Effect of TDZ at various concentrations alone or in combination withNAA on the an shoot length (cm) compared to a combination of KIN and NAA in carnation shoot-tip tures.Vertical bars represent standard error

However, only a slight increase in shoot elongation (0.3cm), was observed in the 4th week in culture (Fig. 6.2, Table 6.2).

Inclusion of TDZ at (0.1, 0.4, and 1.0μM) to the growth medium had an increase in shoot elongation from 1 to 3 weeks in culture, after which no further increase in shoot elongation was observed (Fig. 6.2). Further increase in TDZ concentration to 5.0μM, caused an increase in shoot elongation from 1 to 2 weeks in culture before declining (Fig. 6.2). In the first week, TDZ at various concentrations had significant increase on shoot length, except medium containing 1.0μM TDZ, which did not have a significant increase on shoot length, but was comparable to 5.0μM (Table 6.2). In the 2nd week, TDZ at various concentrations had a significant increase on shoot length, with TDZ at 0.4μM recording a significantly higher ($P \leq 0.001$) shoot length (0.925cm) compared to the rest of the treatments (Table 6.2). During the 3rd week, TDZ at 0.1 and 0.4μM recorded a significantly high increase ($P \leq 0.001$) in shoot elongation (0.85 and 1.0cm), respectively, compared to medium containing TDZ at (1.0 and 5.0μM), which recorded a significantly low increase in shoot elongation (0.6 and 0.55cm), respectively, in the same week of culture (Table 6.2). During the 4th week, TDZ at (0.1 and 0.4)μM maintained a significantly high increase in shoot elongation observed in the previous week (0.85 and 1.0cm), respectively (Table 6.2).

Increasing the TDZ concentration to 1.0μM, did not have a significant increase on shoot elongation over the control, and maintained a low shoot length (0.6cm), similar to that observed the previous week in culture (Table 6.2). However, these results were comparable to those observed in medium consisting of 0.1μM TDZ (Table 6.2). Further increase in TDZ concentration to 5.0μM, also did not significantly increase the shoot length (0.4cm) relative to the control (Table 6.2). Although these results were comparable to that observed in medium containing

1.0μM TDZ, medium amended with 5.0μM TDZ recorded the lowest increase in shoot elongation during the fourth week in culture (Table 6.2).

Medium supplemented with a combination of TDZ+NAA had an increase in shoot elongation over the 4 weeks in culture (Fig. 6.2), and significantly increased ($P \leq 0.001$) the shoot length throughout the culture period compared to MSO medium (Fig. 6.2, Table 6.2). At 3 and 4 weeks in culture, Medium containing a combination of TDZ+NAA recorded significantly high increase in shoot elongation (Table 6.2, Fig. 6.2). Nonetheless, the ratio of TDZ: NAA was more important than the level of concentrations used, hence the similar results obtained in medium containing 0.1μM TDZ +0.01mg L^{-1} NAA and 1.0μM TDZ+0.1mg L^{-1} NAA at 1, 2, 3, and 4 weeks in culture (Table 6.2). Lowering NAA concentration to 0.01mg L^{-1} relative to TDZ concentration at 1.0μM, did not have a significant difference in shoot elongation compared to the other treatments containing a combination of TDZ+NAA during the culture period (Table 6.2). These results were comparable to those observed in medium containing (0.1 and 0.4μM TDZ) (Fig. 6.2, Table 6.2). In addition, there were no significant differences ($P \leq 0.001$) in shoot elongation between treatments containing a combination of TDZ+NAA (Table 6.2).

Medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA had an increase in shoot elongation over the four weeks in culture (Fig. 6.2). In addition, a significant increase in shoot length (0.35, 0.52, 0.85, and 1.175) at (1, 2, 3, and 4) weeks in culture, respectively over the control was observed (Fig. 6.2, Table 6.2). These results were similar to those recorded in medium containing TDZ at (0.1 and 0.4μM) in the 3rd and 4th week in culture, and a combination of TDZ+NAA throughout the culture period (Table 6.2, Fig.6.2).

Table. 6.3 Effect of TDZ at various concentrations alone or in combination with NAA on the mean shoot quality rating[z] in carnation cv. Yair compared to a combination of KIN and NAA.

PGRs	weeks in culture			
	1	2	3	4
MSO	3	3	2.75b	2.25bc
0.1μM TDZ	3	3	3.25ab	3.25ab
0.4μM TDZ	3.5	3.5	3.25ab	3.25ab
1.0μM TDZ	3.25	3.25	3.25ab	3.25ab
5.0μM TDZ	3.5	3.5	2b	2c
0.1μM TDZ+ 0.01mg L^{-1} NAA	3.75	3.25	3.25ab	3abc
1.0μM TDZ+0.01mg L^{-1} NAA	3.5	2.5	2.5b	2.5bc
1.0μM TDZ+0.1mg L^{-1} NAA	3.25	3.25	3ab	3abc
0.2mg L^{-1} KIN+0.2mg L^{-1} NAA	3.75	3.75	4a	4a
Significance	n.s	n.s	**	**
SE	0.4714	0.609	0.419	0.441
CV	13.9	18.9	13.9	15.1
W(Turkey's)	-	-	1.0	1.0

[z] Visual estimates based on colour, and appearance on a scale of 1 to 4 where 1= brown and 4=green and good appearance

n.s, **, = nonsignificant, significant at 0.01 respectively.

Y = Mean separation using Turkey's procedure. Values followed by the same letter in the same column are not significantly different at 5% level of probability.

culture, respectively (Table 6.3). Most of the shoots were pale green to light green, with severe chlorosis and poor growth.

All treatments except medium supplemented with a combination of KIN+NAA had no significant increase on the shoot quality rating relative to the control, in the 3rd and 4th week in culture (Table 6.3). Medium amended with TDZ at 5.0μM had a significantly lower shoot quality rating, compared to medium containing a combination of KIN+NAA (Table 6.3). These results were similar to those observed in medium supplemented with 1.0μM TDZ+0.01mg L^{-1} NAA during the same culture period (Table 6.3). Medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA significantly increased and recorded the highest shoot quality rating (4) at 3 and 4 weeks in culture (Table 6.3). The high shoot quality rating was observed as green, healthy, and vigorous shoots. In brief, a combination of KIN+NAA in the culture medium significantly increased the quality of shoots in the last two weeks of culture. These results were comparable to those recorded in medium amended with TDZ at 0.1, 0.4, and 1.0μM in at 3 and 4 weeks in culture. A higher level of TDZ at concentrations of 5.0μM resulted in significantly low shoot quality rating compared to a combination of KIN+NAA, during the same culture period. These results were similar to those recorded in medium supplemented with a combination of TDZ+NAA at 1.0μM TDZ+0.01mg L^{-1} NAA during the same culture period.

Vitrification rating

Vitrification is a physiological disdorder observed during micropropagation of plants. It is characterised by hyperhydricity of shoot tissues, which appear watery, succulent, and are difficult to handle during subsequent transfers. This condition was scored using a visual estimate based on a scale of 1 to 4: 1=no vitrification (normal

appearance), 2= low vitrification (<50%), 3= high vitrification (>50%), 4= all shoots vitrified.

Highly vitrified shoots were observed in medium supplemented with TDZ at 5.0μM and a combination of TDZ+NAA throughout the culture period (Table 6.4). Shoots with low vitrification were noticed in MSO medium, medium amended with TDZ at 0.1, 0.4, and 1.0μM, and a combination of KIN+NAA (Table 6.4).

The PGRs had a significant effect ($P \leq 0.001$) on vitrification of shoots throughout the culture period (Table. 6.4). Basal medium had no vitrified shoot (1) in the first week of culture (Table 6.4). On the contrary, low vitrification (1.25, 1.25, and 1.5) at (2, 3, and 4) weeks in culture, respectively, was recorded in the same medium (Table 6.4). TDZ at 0.1, 0.4, 1.0, and 5.0μM had a significant increase on the vitrifaction of shoots

in the 1st week in culture (Table 6.4). In addition, medium containing 5.0μM TDZ recorded significantly higher vitrification of shoots compared to medium supplemented with TDZ at (0.1 and 0.4μM), but was similar to medium consisting of 1.0μM TDZ (Table 6.4). These results were similar to those observed in medium supplemented with a combination of TDZ+NAA during the same week (Table 6.4). In the 2nd week, TDZ at (0.1, and 0.4 μM) had no significant Increase on the vitrification of shoots compared to the rest of the medium in relation to the control (Table 6.4). In addition, the results were comparable to those observed in medium consisting of 1.0μM TDZ (Table 6.4). During the same week, TDZ at 5.0μM recorded the highest vitrification of shoot (3.75) (Table 6.4). Similar observations were noticed, in the 3rd and 4th week in culture, with vitrification rating values of (3.75 and 4), respectively

Table. 6.4 Effect of TDZ at various concentrations alone or in combination with NAA on the mean shoot vitrification rating[z] in carnation cv. Yair compared to a combination of KIN and NAA.

PGRs	Weeks in culture			
	1	2	3	4
MSO	1d[y]	1.25e	1.25d	1.5c
0.1µM TDZ	2.25bc	2.25cde	2cd	2c
0.4µM TDZ	2.25bc	2.25cde	2.25bcd	2c
1.0µM TDZ	2.75ab	2.5bcd	2.25bcd	2.25bc
5.0µM TDZ	3.5a	3.75a	3.75a	4a
0.1µM TDZ+ 0.01mg L^{-1} NAA	3ab	3abc	3.25abc	3.25ab
1.0µM TDZ+0.01mg L^{-1} NAA	3.5a	3.5ab	3.5ab	3.5a
1.0µM TDZ+0.1mg L^{-1} NAA	3.5a	3.25abc	3.25abc	3.25ab
0.2mg L^{-1} KIN+0.2mg L^{-1} NAA	1.5cd	1.5dc	1.5d	1.75c
Significance	***	***	***	***
SE	0.481	0.50	0.561	0.43
CV	18.6	19.4	20.5	16.5
W(Turkey's)	1.14	1.19	1.33	1.02

[z] Visual estimates based on watersoaking appearance of tissues on a scale of 1 to 4 where 1=low water soaking and 4=high watersoaking

*** = significant at P<0.001.

[y] = Mean separation using Turkey's procedure. Values followed by the same letter in the same column are not significantly different at 5% level of probability.

(Table 6.4). These results were comparable to those observed in medium supplemented with a combination of TDZ+NAA in the 2nd, 3rd, and 4th week in culture (Table 6.4). There were no significant differences among treatments containing a combination of TDZ+NAA throughout the culture period (Table 6.4).

Medium supplemented with 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA had no significant effect on the vitrification of shoots (1.5, 1.75, 1.5, and 1.75) at (1, 2, 3, and 4) weeks in culture, respectively (Table 6.4). These results were comparable to those observed in medium containing 0.1, 0.4, and 1.0μM TDZ in the 2nd, 3rd, and 4th week in culture (Table 6.4).

In summary, medium supplemented with a combination of KIN+NAA had no significant increase on the vitrification of shoots relative to the control. In addition, comparable results were observed in medium containing TDZ at (0.1, 0.4, and 1.0μM) in the 2nd , 3rd , and 4th week in culture. However, increasing the TDZ concentration to 5.0μM significantly increased the vitrification of shoots compared to a combination of KIN+NAA, throughout the culture period.

Rooting and establishment in sterile soil of carnation cv. Yair shoots

Shoots transferred from medium containing 1.0μM TDZ easily rooted in MSO medium (Plate. 10: right). Shoots also rooted directly in sterile soil after transfer from medium containing 1.0μM TDZ (Plate 11). The plantlets were acclimatized using simple clear polyethylene sleeves (Plate. 12) and established in soil after 4 weeks (Plate. 13).

Effect of dose and duration of exposure to TDZ on the mean number of shoots, mean shoot length, Mean shoot quality rating, and virtification in *Dianthus caryophillus* cv Yair.

Number of shoots per explant

Both the level and duration of exposure to TDZ had a significant effect ($P \leq 0.001$) on the number of shoots (Table. 6.5). There were significant interactions ($P \leq 0.1$) between the dose and duration of exposure to TDZ on the number of shoots (Table. 6.5). Significant quadratic relationships were observed between the concentration of TDZ applied at various duration of exposure and the number of shoots (Fig. 6.3).

The number of shoots increased as the concentration of TDZ increased at each duration of exposure (Fig. 6.3). A duration of exposure to TDZ for only 3 d resulted in an appreciable number of shoots (16.58) although at relatively higher TDZ concentrations (5.0μM) compared to prolonged exposures of >40 d (12.88). A 10 d exposure to 5.0μM TDZ resulted in the highest number of shoots (25.08) (Table 6.5, Fig. 6.3). Prolonged exposures (24 and >40 d) to high levels of TDZ at 5.0μM, reduced the number of shoots (19.67 and 16.25), respectively compared to 10 d exposure (Table 6.5, Fig. 7.3). Longer duration of exposure to TDZ at lower concentrations (0.1 and 0.4μM), resulted in an increase in the number of shoots, but, was lower than that observed at 5.0μM TDZ for a duration of exposure for 3 d (Table 6.5, Fig. 6.3). The efficacy of TDZ was less under a duration of exposure for 3 d and highest at 10 d (Fig. 6.3).

Table 6.5 Effect of level and duration of exposure to TDZ in days on the mean number of shoots per explant in carnation cv. Yair shoot-tip cultures

Concentration of TDZ in μM	Duration of exposure to TDZ in days				
	3	10	24	>40	Means
0.1	4.5a[y]	6b	7.08a	7.75a	6.33
0.4	5.75a	10.25ab	9.92a	10.83a	9.12
1.0	8.83a	15.67ab	17.17a	16.67a	13.96
5.0	16.58a	25.08a	19.67a	16.25a	19.48
Means	8.92	14.25	13.46	12.88	12.22
Duration of exposure to TDZ		***			
Concentration of TDZ		***			
Exposure * Concentration Interaction		*			

*, *** = significant at $P \leq 0.05$ and 0.001 level of probability, respectively

[y] = Mean separation within columns by Turkey's procedure at 0.05% level of probability.

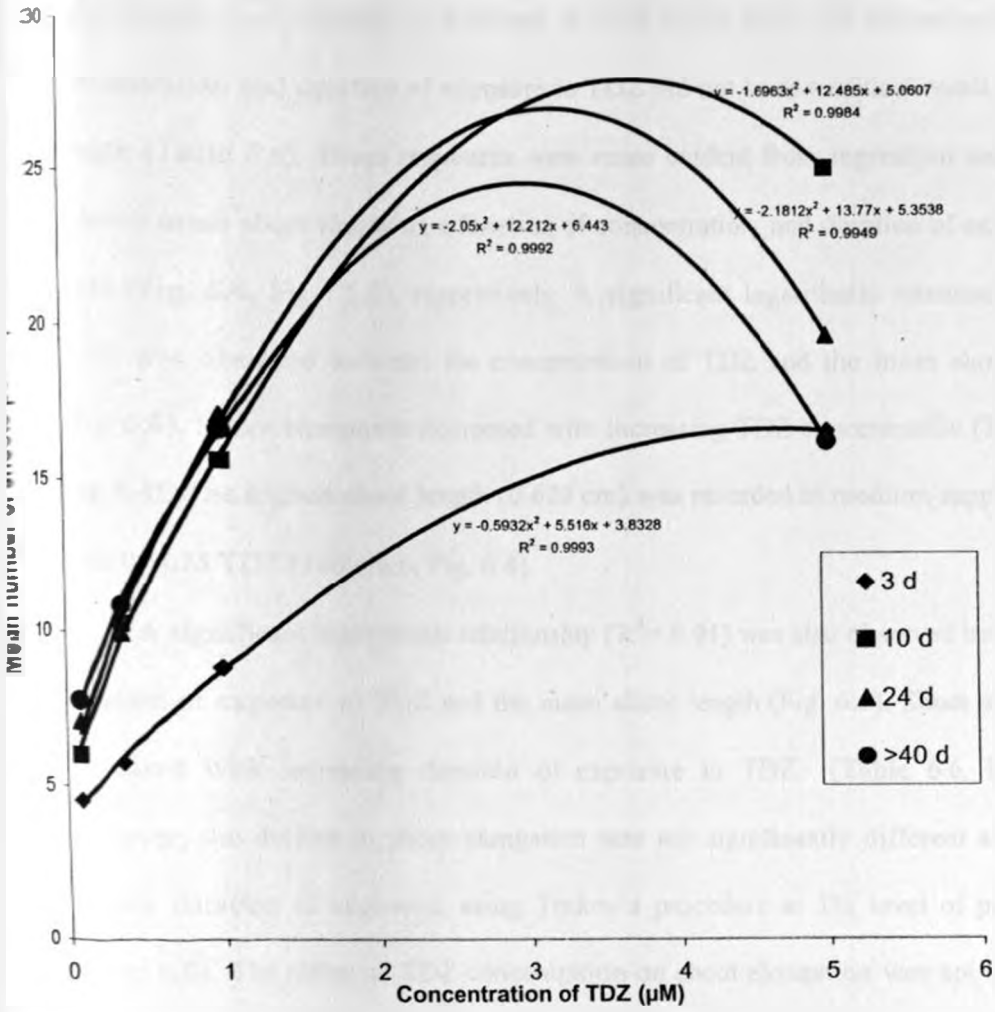


Fig. 6.3 Effect of concentration and duration of exposure to TDZ in days on the mean number of shoots in carnation cv. Yair shoot-tip cultures

Duration of exposure in days to TDZ

Shoot length

The elongation of shoots significantly differed ($P \leq 0.001$) between the concentration and duration of exposure to TDZ (Table 6.6). The interaction between concentration and duration of exposure to TDZ did not have a critical result on shoot length (Table 6.6). These responses were more evident from regression analyses of relative mean shoot length as a function of concentration, and duration of exposure to TDZ (Fig. 6.4, Fig. 6.5), respectively. A significant logarithmic relationship (R^2= 0.96) was observed between the concentration of TDZ and the mean shoot length (Fig. 6.4). Shoot elongation decreased with increasing TDZ concentration (Table 6.6, Fig. 6.4). The highest shoot length (0.629 cm) was recorded in medium supplemented with 0.1 μM TDZ (Table 6.6, Fig. 6.4).

A significant logarithmic relationship (R^2= 0.91) was also observed between the duration of exposure to TDZ and the mean shoot length (Fig. 6.5). Shoot elongation decreased with increasing duration of exposure to TDZ (Table 6.6, Fig. 6.5). However, the decline in shoot elongation was not significantly different among the various duration of exposure, using Turkey's procedure at 5% level of probability (Table 6.6). The effect of TDZ concentration on shoot elongation was not dependent on the duration of exposure.

Table. 6.6 Effect of dose and duration of exposure to TDZ on the mean shoot length (cm) in carnation cv. Yair shoot-tip cultures.

Concentration of TDZ in µM	Duration of exposure to TDZ in days				
	3	10	24	>40	Means
0.1	0.767	0.683	0.525	0.542	0.629a[y]
0.4	0.642	0.600	0.458	0.458	0.540ab
1.0	0.650	0.542	0.375	0.458	0.506ab
5.0	0.392	0.308	0.317	0.308	0.331b
Means	0.613a	0.533a	0.419a	0.442a	
Duration of exposure to TDZ		***			
Concentration of TDZ		***			
Exposure * Concentration Interaction		ns			

ns, *** = nonsignificant, and significant at $P \leq 0.001$

[y] = Mean separation using Turkey's procedure at 0.05% level of probability

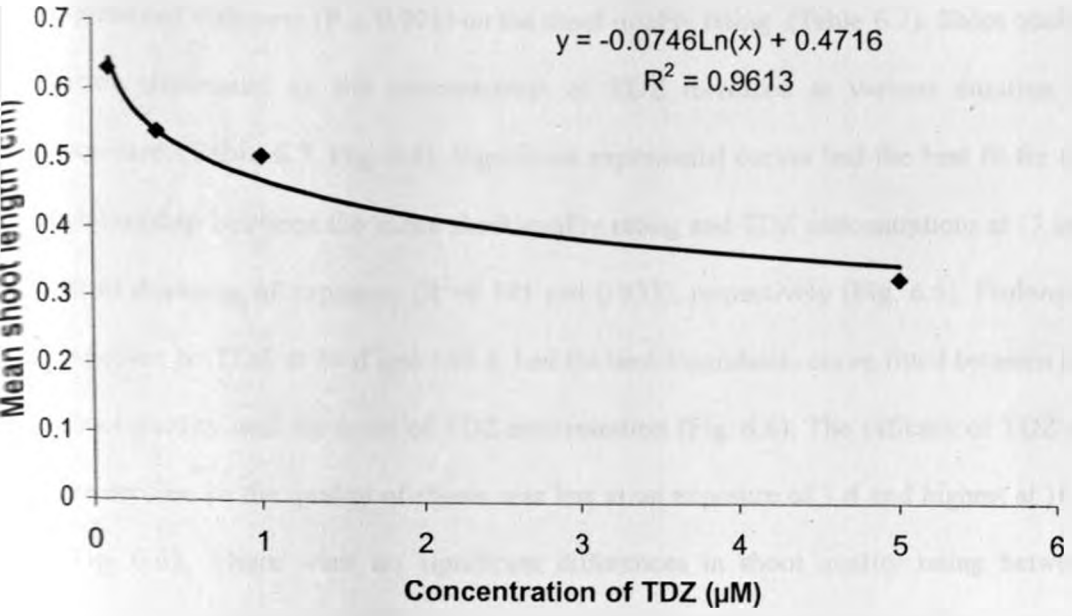


Fig.6.4 Effect of TDZ concentration on the mean shoot length (cm) in carnation cv. Yair shoot-tip cultures

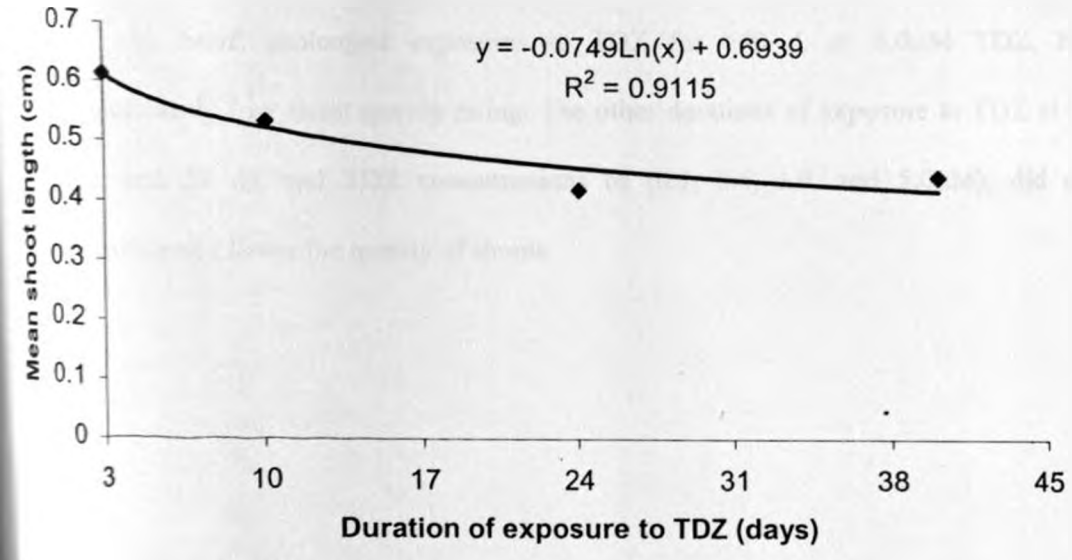


Fig.6.5 Effect of duration of exposure to TDZ on the mean shoot length (cm) in carnation cv. Yair shoot-tip cultures

Shoot quality rating

The concentration of TDZ, the duration of exposure, and their interaction had a significant outcome ($P \leq 0.001$) on the shoot quality rating (Table 6.7). Shoot quality rating decreased as the concentration of TDZ increased at various duration of exposure (Table 6.7, Fig. 6.6). Significant exponential curves had the best fit for the relationship between the mean shoot quality rating and TDZ concentrations at (3 and 10 d) duration of exposure (R^2=0.743 and 0.932), respectively (Fig. 6.6). Prolonged exposure to TDZ at 24 d and >40 d, had the best logarithmic curve fitted between the shoot quality and the level of TDZ concentration (Fig. 6.6). The efficacy of TDZ on the decline in the quality of shoots was less at an exposure of 3 d and highest at 10 d (Fig. 6.6). There were no significant differences in shoot quality rating between medium supplemented with TDZ at various concentrations and exposed for 3, 10, 24, and >40 d, except treatments containing 5.0μM TDZ (1.75) for a duration of exposure for >40 d to TDZ (Table 6.7).

In brief, prolonged exposures to TDZ for >40 d, at 5.0μM TDZ, had significantly low shoot quality rating. The other durations of exposure to TDZ at (3, 10, and 24 d), and TDZ concentrations of (0.1, 0.4, 1.0, and 5.0μM), did not significantly lower the quality of shoots.

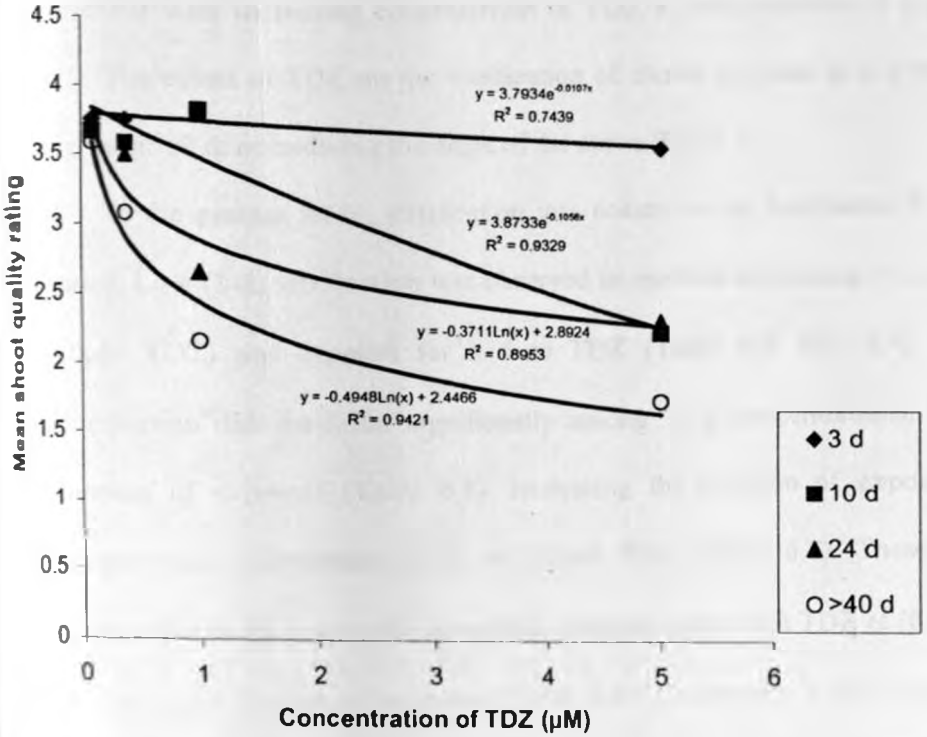
Table. 6.7 Effect of dose and duration of exposure to TDZ on the mean shoot quality rating[z] in carnation cv. Yair shoot tip cultures.

Concentration of TDZ in µM	Duration of exposure to TDZ in days				
	3	10	24	>40	Means
0.1	3.75a[y]	3.667a	3.667a	3.583a	3.667
0.4	3.75a	3.583a	3.5a	3.083a	3.479
1.0	3.833a	3.833a	2.667a	2.167a	3.125
5.0	3.583a	2.25a	2.333a	1.75b	2.479
Means	3.729	3.33	3.042	2.646	3.188
Duration of exposure to TDZ		***			
Concentration of TDZ		***			
Exposure * Concentration Interaction		***			

[z] = Scores were visual attributes rated on a 1-4 scale; 1 = brown, poor growth; 2 = pale green, severe chlorosis, fair to poor growth; 3 = light green, slight chlorosis, good growth; 4 = green, healthy and vigorous growth.

***= significant at 0.001 level of probability

[y] = Mean separation using Turkey's procedure. Values followed by the same letter within columns are not significantly different at 5% level of probability.



6.6 Effect of concentration and duration of exposure to TDZ on the mean shoot lity rating in carnation cv. Yair shoot-tip cultures

= Duration of exposure in days to TDZ

Vitrification rating

The level of TDZ, duration of exposure and their interaction had a significant effect ($P \leq 0.001$) on vitrification of shoots (Table. 6.8). Vitrification of shoots increased with increasing concentration of TDZ at each duration of exposure (Fig. 6.7). The effect of TDZ on the vitrification of shoots was less at 3 d exposure, and highest at 10 d, considering the slope of the curve (Fig. 6.7).

In the present study, vitrification was noticed in all treatments, but the degree varied. Low (1-2) vitrification was observed in medium containing (0.1, 0.4, 1.0, and 5.0μM TDZ) and exposed for 3 d to TDZ (Table 6.8, Fig. 6.7). In addition,. Vitrification did not differ significantly among TDZ concentrations, for the same duration of exposure (Table 6.8). Increasing the duration of exposure to 10 d, recorded low vitrification (2.16) at 0.1μM TDZ (Table 6.8). These results were comparable to those recorded in medium supplemented with TDZ at (0.4 and 1.0μM) for the same duration of exposure (Table 6.8). Conversely, higher concentration of TDZ at 5.0μM, significantly increased and recorded the highest vitrification of shoots at 10 d exposure (Table 6.8, Fig. 6.7). Prolonged exposure for (24 and >40 d), recorded significantly high vitrification of shoots at 0.1, 0.4, 1.0, and 5.0μM TDZ (Table 6.8, Fig. 6.7).

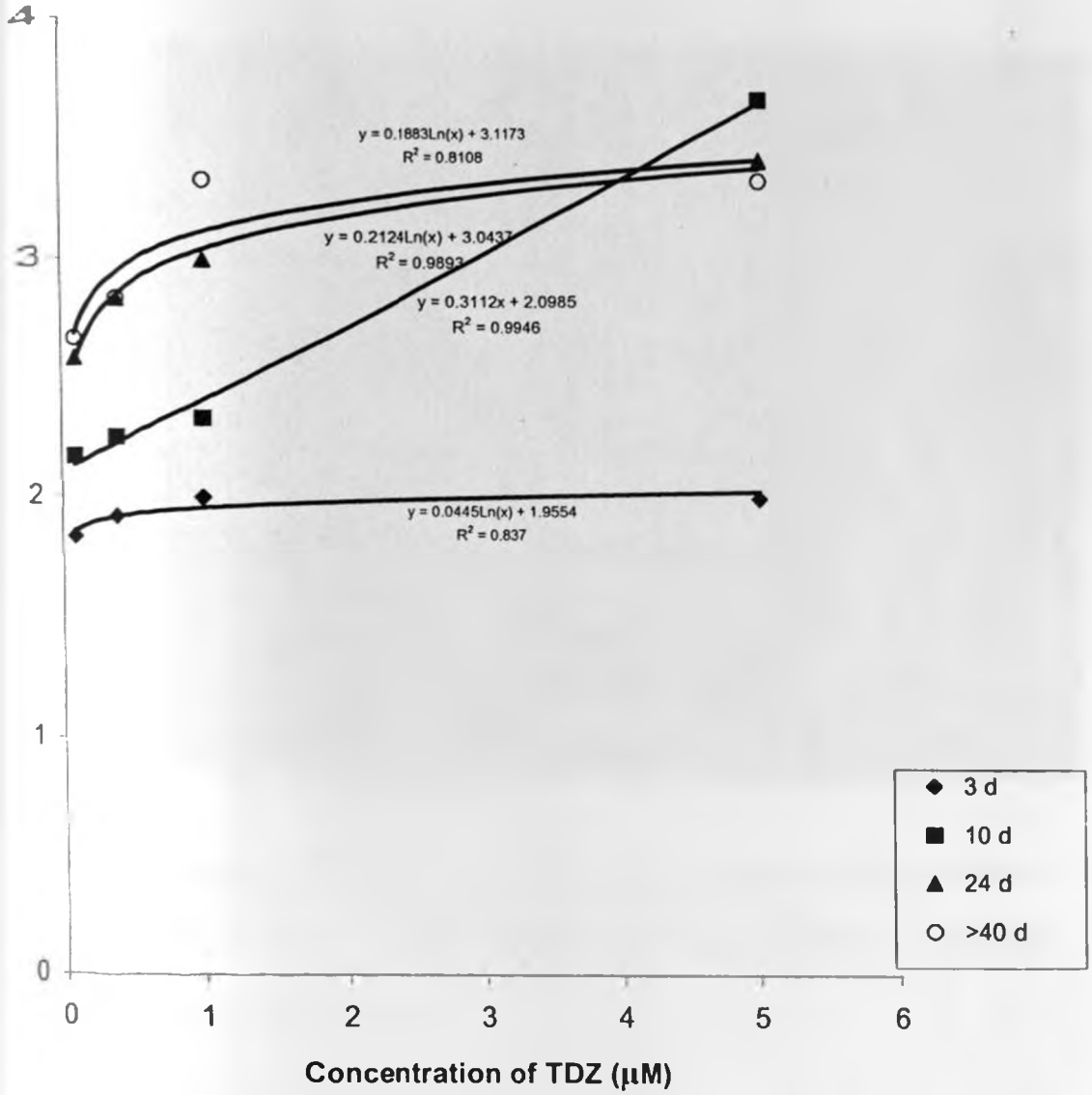
In conclusion, Low vitrification was observed in medium containing TDZ at various concentrations and exposed for 3 d. Increasing the duration of exposure to 10 d had similar results, except medium amended with 5.0μM TDZ, which recorded a significantly high vitrification of shoots. Prolonged exposure for 24 and >40 d, significantly increased the vitrification of shoots at all the TDZ concentrations used.

Table. 6.8 Effect of dose and duration of exposure to TDZ on the mean vitrification[z] rating of shoots in carnation cv. Yair shoot-tip cultures

Concentration of TDZ in µM	Duration of exposure to TDZ in days				
	3	10	24	>40	Means
0.1	1.833a	2.167b	2.583a	2.667a	2.313
0.4	1.917a	2.25ab	2.833a	2.833a	2.458
1.0	2.0a	2.333ab	3.0a	3.333a	2.667
5.0	2.0a	3.667a	3.417a	3.333a	3.104
Means	1.937	2.604	2.958	3.042	2.635
Duration of exposure to TDZ		***			
Concentration of TDZ		***			
Exposure * Concentration Interaction		***			

[z] Scores were rated on a 1-4 scale; 1 = no vitrified shoots 2 = low vitrification of shoots, < 50%; 3 = high vitrification of shoots, ≥ 50%; 4 = all shoots vitrified

[y] = Mean separation using Turkey's procedure. Values followed by the same letter within columns are not significantly different at 5% level of probability.



6.7 Effect of concentration and duration of exposure to TDZ on the mean vitrification ng in carnation cv. Yair shoot-tip cultures

Duration of exposure in days to TDZ

Plate 9 Formation of friable to compact, pink to green callus in medium consisting of 5.0μM TDZ, for an exposure of 10 days from *Dianthus caryophillus* cv. Yair shoot-tip cultures at 2-3 weeks in culture.

Plate 10 Left: multiple shoots induced in medium containing 1.0μM TDZ and transferred to MSO medium at 6 weeks subculture. Right: rooted *Dianthus caryophillus* cv. Yair shoots in MSO medium at 10 weeks of culture.

Plate 11 *Dianthus caryophillus* plantlets transferred from medium with 1.0μM TDZ, directly to sterile soil at 1 week.

Plate 12 Acclimation of *Dianthus caryophillus* cv. 'Yair' plantlets using simple clear polyethylene sleeves at 7 to 28 d after transfer to sterile soil.

Plate 13 Established *Dianthus caryophillus* cv. Yair plantlets in soil at 4 weeks after transfer to sterile soil and before pinching.

Discussions

Establishment

An inverse relationship was observed between the number of shoots and shoot elongation. Cultures with high number of shoots also had highly vitrified shoots with poor shoot quality rating. Vitrification was inversely correlated with shoot quality rating.

Number of shoots per explant

The study investigated the effect of TDZ alone at various concentrations or in combination with NAA at relatively low levels (0.1 and 0.01mg/l), compared to a combination of 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA as previously compiled from published protocols by several scientists (George and Sherrington, 1984; Zimmerman *et al.*, 1986). Shoot regeneration in medium containing TDZ increased with increasing level of TDZ, and TDZ at 5.0μM recorded the highest number of shoots compared to a combination of KIN+NAA. These results are consistent with those reported by Watad *et al.* (1996), who demonstrated that TDZ induced regeneration of shoots from carnation cv. White Sims stem segments with an efficiency which increased with increase in TDZ concentration ranging from 0.25 to 18mg/l. Similarly, Murthy *et al.* (1998) reviewed studies on TDZ mediated shoot regeneration in many plant systems, and reported that high efficiency was achieved by TDZ compared to other phytohormones. The exact mode of action for TDZ is not yet known, however, It is thought to act directly as a cytokinin (Mok *et al.*, 1982; Mok and Mok, 1985) and or through the modulation of endogenous growth substances (Hutchinson *et al.*, 1996a). These findings could explain the high efficiency observed in TDZ-mediated shoot regeneration from carnation cv. Yair shoot-tip cultures. In the present study, we report the possibility of TDZ substituting for auxin and cytokinin requirement for shoot

regeneration from carnation cv. Yair shoot-tip cultures, with an even higher efficiency. Similar observations have been demonstrated in geranium hypocotyl explants during somatic embryogenesis (Gill *et al.*, 1993; Visser *et al.*, 1992; Hutchinson *et al.*, 1996a; Murthy and Saxena, 1998).

At 5.0μM TDZ, friable to compact pink to green callus was observed from the 3rd week in culture. Callus induction is mediated by auxins or a combination of high auxin: cytokinin ratio in the growth medium (Skoog and Schmitz, 1979). TDZ has been reported to induce callus in cotton leaf disks (Jayashankar *et al.*, 1991), and Grape bud cuttings (Lin *et al.*, 1989) under in vitro conditions. In the present study, we report for the first time callus induction in carnation cv. Yair shoot-tip cultures in medium supplemented with higher levels of TDZ at 5.0μM

Addition of NAA to Medium containing TDZ resulted in the lowest number of shoots compared to TDZ at various concentrations and a combination of KIN+NAA. Probably due to a greater shift in plant growth substances towards high auxin levels which have been reported to enhance shoot elongation as opposed to multiplication (Vanderhoef, 1980). These findings can be supported by the observation that high TDZ levels induced elevations of endogenous growth substances, and auxins may have been included (Hutchinson *et al.*, 1996a; Murthy *et al.*, 1998). We could also speculate that a combination of TDZ+NAA did not result in high proliferation of shoots, probably due to the decreased cytokinin-like effect of TDZ in the presence of NAA.

Medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA significantly increased the number of shoots, which were comparable to those observed in medium containing lower levels of TDZ at (0.1 and 0.4) μM during the same culture period. Suggesting

that TDZ at even lower levels produced similar results to those observed in previously compiled protocols (George and Sherrington, 1984; Zimmerman *et al.*, 1986).

Medium supplemented with 0.2mg L^{-1} KIN or 0.2mg L^{-1} NAA alone, was found to be ineffective in the regeneration of shoots from shoot-tip explants, which turned brown and died after 3 to 5 days in culture. Previously, Mujib and Pal (1994), showed poor growth of shoot-tip explants in the presence of KIN (0.5mg L^{-1}) alone in carnation cv. William Sim.

Although shoot-tip explants survived in MSO medium throughout the four weeks in culture, the multiplication of shoots was not observed. Suggesting the importance of phytohormones in regulating in vitro regeneration of shoots (Janicks, 1986; Skoog and Miller, 1957; Skoog and Schmitz, 1979). A time lag of one week was observed before the treatments recorded a significant effect on the number of shoots. Suggesting a possibility of metabolic processes occurring before growth responses are observed.

In summary, TDZ at 5.0μM was more efficient in regeneration of shoots from carnation cv. Yair shoot-tip explants, compared to previously published protocols consisting of a combination of 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA. Suggesting a possibility of TDZ substituting for auxin and cytokinin requirements for shoot regeneration from shoot-tip explants.

Shoot length

The absence of a time lag before a response to PGRs was noticed on shoot elongation, suggests that shoot elongation is very sensitive to the presence of PGRs in the medium.

A clear difference between treatments in shoot elongation was observed during the last two weeks of the establishment phase. Appreciable shoot elongation was

observed in medium supplemented with lower TDZ levels at (0.1, 0.4 and 1.0μM). Increasing the TDZ concentration to 5.0μM resulted in low shoot elongation, especially in the last week of culture, during the establishment phase. These results are similar to those reported in maize, oats, radish etc (Devlin *et al.*, 1989). These authors demonstrated that TDZ had inhibitory effects on shoot elongation in maize, oats, radish etc. Pountney and Swietlik (1988), observed inhibition of shoot growth in sour orange. Similar observations were reported by Huetteman and Preece, (1993) and Lu (1993), who showed that TDZ at supra-optimal levels caused poor shoot elongation in in-vitro cultures among other disorders. Likewise, results reported in a previous study with tuberose (data unpublished) support these findings. Wherein, TDZ at 5.0μM TDZ had inhibitory effects on shoot elongation.

Low shoot elongation in medium containing 5.0μM TDZ could be due to inhibitory effects of TDZ at high levels (Murthy *et al.*, 1998), and or competition for nutrients as a result of high regeneration of shoots (Salisbury and Ross, 1991). Low shoot elongation could be attributed to TDZ having cytokinin-like effects (Mok *et al.*, 1982). Cytokinins have been reported to inhibit shoot elongation, the effect being more pronounced at increased concentrations (Economou and Spanoudaki, 1986; Vanderhoef, 1973). In addition, ethylene effects on shoot elongation could also be expected to cause the low shoot elongation observed at high TDZ concentration. Ethylene has been reported to inhibit shoot elongation, and cause thickening and shortening of stems (Beyer *et al.*, 1984). Further evidence for shortening of shoots by TDZ at high concentration, can be explained through the possibility of TDZ influencing the metabolism of gibberellines, as was demonstared by Lu (1993), in TDZ mediated adventitious shoot regeneration of woody plants. Conversely, relatively high shoot elongation in medium containing 0.1 and 0.4μM TDZ could be

due to reduced inhibitory effects of TDZ at lower concentrations, and or reduced competition for nutrients as a result of lower regeneration of shoots.

The higher the number of shoots, the shorter the shoot length. This could be as a result of competition for nutrients and also may be due to the dwarfing effect of higher levels of TDZ, mimicking cytokinin effects, or due to elevated, TDZ induced ethylene production (Hutchinson *et al.*, 1996a, 1997a; Murthy *et al.*, 1998), resulting in inhibition of shoot elongation.

Medium supplemented with a combination of TDZ+NAA recorded longer shoots, which were comparable to those observed in medium supplemented with TDZ at (0.1 and 0.4μM) and a combination of KIN+NAA in the last two weeks in culture. Probably as a result of reduced competition due to the regeneration of less number of shoots and or the effect of NAA and endogenous auxins, which may have been stimulated by TDZ. Hutchinson *et al.* (1996b), demonstrated that TDZ induced elevation of endogenous growth substances including auxins. Auxins have been reported to cause shoot elongation as opposed to shoot multiplication (Janicks, 1989; Vanderhoef, 1980). Similarly, Sutter (1986) reported shoot elongation from buds developed on transversely cut halves of corms when transferred to medium containing NAA at 0.05mg/l in *Gladiolus* x *Homoglossum* hybrid. These findings could explain the relatively longer shoots observed in medium consisting of a combination of TDZ+NAA.

Medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA had longer shoots and they were comparable to those observed in medium containing a combination of TDZ+NAA and TDZ at (0.1 and 0.4μM). Suggesting that medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA could have provided optimum conditions for shoot elongation compared to TDZ at higher levels (1.0 and 5.0μM). Besides, fewer shoots

were also recorded in medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA, and low competition for nutrients may have resulted in increased shoot elongation. (Janicks, 1989).

Basal medium did not support shoot elongation during most of the weeks in culture, except the 4th week, when a very slight increase was observed. Again pointing to the important role played by phytohormones in regulating shoot elongation (Vanderhoef, 1980).

In brief, satisfactory shoot elongation was achieved in TDZ medium at (0.1, 0.4, and 1.0μM), at the end of the establishment phase, which was similar to that observed in medium containing a combination of KIN+NAA as reported in previous protocols. Our results propose that higher levels of TDZ at 5.0μM should be avoided, due to the poor shoot elongation observed, which interferes with handling during subsequent transfers.

Shoot quality rating

Medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA significantly increased, and recorded the highest shoot quality at 3 and 4 weeks in culture. Good quality shoots were observed in medium containing 0.2mg/l KIN/0.2mg/l NAA, probably as a result of the creation of an optimum balance in growth substances (mainly auxins and cytokinins) as was reported by Skoog and Miller (1957).

During the first two weeks in culture, the PGRs did not significantly affect the shoot quality rating. Suggesting a time lag of 2 weeks before a significant effect on the shoot quality was realised. However, the shoot quality was good (3 to 3.75), which was characterised by light green to green colour, slight chlorosis, and good growth, during the same culture period. Plants have been shown to contain endogenous growth substances, which support growth and development (Salisbury and Ross,

1991). This could explain why the shoots had good quality despite the PGRs not having a significant influence on the quality of shoots. The good quality of shoots observed in the first 2 weeks of culture also suggest that other factors, in addition to PGRs could be involved in influencing the quality of shoots. Preece (1995) and Ziv (1991), showed that, the quality of shoots may also be influenced by the composition and concentration of salts in the nutrient medium, and that nutrient salts in the medium could partially substitute for PGR and vise versa in causing a particular physiological response in plants (Preece, 1995; Ziv, 1991). Probably the nutrient salts in the medium substituted partially for PGRs and supported good quality shoots. Castillo *et al.* (1997), demonstrated that irradiation and iron chelet source influenced the quality of shoots in *carica pap*aya L. These findings further suggest that shoot quality could be influenced by light.

In the 3rd and 4th weeks in culture, TDZ at various concentrations had no significant increase on the quality of shoots in relation to the control. However, the shoot quality rating in medium containing TDZ at (0.1, 0.4, and 1.0μM) was good and comparable to that observed in medium containing a combination of KIN+NAA. Perhaps due to other factors contributing to increased shoot quality rating. Preece (1995) and Ziv (1991) reported that shoot quality was influenced by the type and concentration of nutrient salts used in the growth medium, in addition to the type and concentration of PGRs included in the culture medium. Preece (1995), showed that nutrient salts could substitute for PGR requirements in a number of plants. In the present study, nutrient salts seem to have contributed more to increased shoot quality rating in medium consisting TDZ at (0.1, 0.4, and 1.0μM). Further evidence is provided by the fair to good shoot quality observed in MSO medium. The lack of significane in TDZ increasing shoot quality could be attributed to the negative effects

A scenario which probably may not have occurred in medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA.

Basal medium did not support shoot elongation during most of the weeks in culture, except the 4th week, when a very slight increase was observed. Again pointing to the important role-played by phytohormones in regulating shoot elongation (Vanderhoef, 1980).

It can be concluded that, although TDZ at lower levels (0.1, 0.4, and 1.0μM) did not significantly increase the quality of shoots, good quality shoots which were comparable to those observed in medium containing a combination of KIN and NAA were noticed. Suggesting the possibility of nutrient salts and probably light in contributing to good shoot quality. However, increasing the TDZ concentration to 5.0μM resulted in poor quality shoots. Probably as a result of TDZ-induced accumulation of ethylene, which could have lowered the quality of shoots. However, TDZ at various concentrations and in combination with NAA did not significantly increase the quality of shoots, although generally, good shoot quality was observed. Suggesting the important role-played by the interaction between nutrient salts and PGRs in supporting good shoot quality.

Vitrification

Basal medium had low vitrification of shoots, suggesting that the medium used provided optimum conditions, which did not enhance the vitrification of shoots. Similar results were reported in medium containing 0.2mg L^{-1} KIN+0.2mg L^{-1} NAA throughout the culture period. Low TDZ concentrations at (0.1, 0.4, and 1.0μM) had significantly low increase in the vitrification of shoots. Increasing TDZ concentration to 5.0μM significantly increased the vitrification of shoots. The vitrification of shoots is a phenomenon characterized by a translucent, watersoaked, succulent, and watery

appearance that can result in deterioration, failure to proliferate, and poor acclimation of plantlets (Hartmann and Kiester 1985; Kyte, 1990). Vitrification is thought to be caused by an upset in osmotic potential, probably as a result of the interaction between PGRs and nutrient salts in the medium (Kyte, 1990; Preece, 1995; Ziv, 1991). High levels of cytokinin in the culture medium have been reported to increase the vitrification of shoots (Kyte, 1990; Pierik, 1987). TDZ has been reported to act like a cytokinin, directly (Mok *et al.*, 1982), and or through the modulation of endogenous cytokinins (Capelle *et al.*, 1983). We could speculate that TDZ at relatively higher concentrations of 5.0μM may have mimicked cytokinins and increased the vitrification of shoots. The high vitrification caused by TDZ at higher levels may also be due to a disruption in osmotic potential resulting from the accumulation of mineral ions and other stress related metabolites (Murch *et al.*, 1997; Murch and Saxena, 1997).

Medium containing a combination of TDZ+NAA significantly increased the vitrification of shoots throughout the culture period, and the results were comparable to those reported in medium supplemented with TDZ at higher concentration (5.0μM). Caboni *et al.* (1996), demonstrated that inclusion of 0.1μM TDZ and 2.7μM NAA into the culture medium, resulted in vitrified shoots of apple root stock 'Jork 9'. Perhaps as a result of a shift towards elevated auxin levels, caused by both the presence of NAA and TDZ in the medium. High levels of PGRs (auxins and cytokinins) in the medium may cause an increase in the vitrification of shoots, as was demonstrated by (Ziv, 1991). The possibility of salts in the nutrient medium contributing to the vitrification of shoots cannot be ruled out. This is because vitrification has been reported to be a physiological disorder caused by an interaction of nutrient salts and PGRs in the medium (Preece, 1995; Ziv, 1991).

In summary, TDZ at lower concentrations (0.1, 0.4, and 1.0μM) did not significantly increase the vitrification of shoots, and similar observations were noticed in medium containing a combination of KIN+NAA. However, higher levels of TDZ at 5.0μM significantly increased the vitrification of shoots. These results were similar to those observed in medium consisting of TDZ+NAA. It seems, inclusion of NAA to TDZ medium did not lower the vitrification of shoots and instead increased it.

Effect of dose and duration of exposure to TDZ on the mean number of shoots, mean shoot length, Mean shoot quality rating, and virtification.

Number of shoots

Both the concentration and duration of exposure to TDZ had a significant influence on the number of shoots. The effect of TDZ concentration on the number of shoots was dependent on the duration of exposure. The number of shoots increased at a decreasing rate with increase in TDZ concentrations. Significant quadratic relationships were observed between TDZ concentrations at various duration of exposure and the number of shoots. A duration of exposure for 3 d had an appreciable number of shoots, although this was achieved at higher TDZ concentration of 5.0μM. In addition, TDZ was more efficient at a duration of exposure for 10 d, and recorded the highest number of shoots at 5.0μM TDZ. TDZ has been reported to induce morphogenic responses with an efficiency comparable to or higher than that of other phytohormones, at relatively lower concentrations for a shorter duration of exposure (Hutchinson and Saxena, 1996a; Preece *et al.*, 1991; Visser *et al.*, 1992). Indicating that TDZ is a potent growth regulator.

The high efficiency in regeneration of shoots achieved by TDZ could be attributed to it's ability to mimic cytokinin-like effects (Mok *et al.*, 1982), and or modulate endogenous growth substances (Hutchinson et al 1996a). Prolonged exposures for (24 and >40 d), inhibited the multiplication of shoots, especially at 5.0μM TDZ. These results corroborate earlier work by Visser *et al.* (1992), which demonstrated that TDZ at higher concentration of 5.0μM and prolonged exposures inhibited the formation of somatic embryos in geranium hypocotyls explants. Similarly, Hutchinson (1996), used TDZ at 10μM for a duration of exposure for 3 d and successfully achieved somatic embryogenesis in geranium hypocotyls cultures..

However, in another study, higher TDZ concentration of 10μM with explants continously exposed to TDZ was toxic to the geranium hypocotyl explants and no somatic embryos were observed (Hutchinson *et al.*, 1996a).

The above findings suggest that, TDZ is a potent PGR and at only short duration of exposure, cause significant morphogenic responses in plants under in vitro conditions. However, prolonged exposures were detrimental to the morphogenic responses. Probably as a result of negative effects observed when the concentration of PGRs is increased resulting from negative feefback response, and or accumulation of growth substances with negative effect on morphogenic responses such as ethylene. We demonstrate for the first time the effect of concentration and duration of exposure to TDZ on the number of shoots in carnation cv. Yair shoot-tip cultures.

Shoot length

Generally, the higher the number of shoots, the shorter the shoot length. Shoot length was significantly influenced by the concentration and the duration of exposure to TDZ. However, the effect of TDZ concentration on shoot elongation was not influenced by the duration of exposure. Shoot length decreased with increasing concentration of TDZ. This response was better shown by a significant logarithmic relationship. TDZ at supra-optimal concentrations has been reported to inhibit shoot elongation (Huetteman and Preece, 1993; Lu, 1993). This could be due to competition resulting from an increased number of shoots with increasing TDZ concenration. In this case, TDZ mimicked cytokinin activity by enhancing shoot proliferation compared to shoot elongation and also inhibiting shoot elongation at higher concentrations, a response similar to cytokinins. TDZ may have induced the elevation of ethylene, which inhibit shoot elongation (Beyer *et al.*, 1984). The possibility of TDZ being involved in the metabolism of gibberellins to influence shoot elongation

can not be ignored, as was demonstrated by (Lu *et al.*, 1993). They showed that regenerants originating from TDZ-induced adventitious shoots in woody plants often tend to be dwarf with shortened internodes. These findings could possibly explain the decrease in shoot elongation with increasing TDZ concentration or duration of exposure.

Shoot length decreased with increasing duration of exposure to TDZ. This response was better shown by a significant logarithmic relationship. Suggesting that prolonged exposure had a similar effect on the cultures as increased concentration, resulting in negative responses explained above.

In the present study, we demonstrate for the first time the effect of concentration and duration of exposure to TDZ on shoot elongation in carnation cv. Yair shoot-tip cultures. Competition for nutrients could be one of the factors influencing shoot elongation. In addition, TDZ having cytokinin-like activities might have promoted shoot proliferation instead of shoot elongation at higher levels (Bruce and Kefford, 1965; Malik and Saxena, 1992a). Present results suggest that shoot length is not affected by an interaction of level and duration of exposure, in other words, there was no interaction and each factor may have acted independently. To obtain desirable shoot length, the level of TDZ is important. Prolonged exposure to TDZ had a dwarfing effect on shoots. This characteristic has been reported with Cytokinins at supra-optimal levels and ethylene (Salisbury and Ross, 1991).

Shoot quality rating

The level of TDZ had a significant effect on the shoot quality rating. Likewise, the duration of exposure to TDZ had a notable effect on the shoot quality rating during the culture period. The effect of TDZ concentration on the shoot quality rating was dependent on the duration of exposure. Shoot quality decreased with increasing

TDZ concentration at each duration of exposure. High shoot quality rating was observed in TDZ medium at various concentrations for a duration of exposure for 3, 10, 24 and >40 d, except in medium supplemented with 5.0μM TDZ and exposed for >40 d, which significantly lowered the quality of shoots. Some of the cytokinin-like effects of TDZ include retention of chlorophyll (Bruce *et al.*, 1965; Mok *et al.*, 1982; Visser *et al.*, 1995). TDZ has been reported to cause retention of chlorophyll, which may explain the high shoot quality observed in most of the cultures. However, high concentrations for prolonged exposures to TDZ could have resulted into an increase in endogenous growth substances some of which have negative effect on the quality of shoots. Ethylene has been reported to cause yellowing of tissues, an indication of degradation of chlorophyll (Beyer *et al.*, 1984). These results could explain the poor quality of shoots observed in medium containing high levels of TDZ at 5.0μM and exposed for longer days to TDZ (>40 d).

In this regard, we can conclude that other factors, such as nutrient salts in the medium and light, in addition to TDZ may also have contributed to the quality of shoots, as was reported by Caboni *et al.* (1997), Preece (1995), and Ziv (1991).

Vitrification

The level of TDZ, duration of exposure to TDZ and their interaction had a significant effect on the vitrification of shoots. Low vitrification was observed in medium supplemented with TDZ at various concentrations for a shorter duration of exposure for 3 d. Increasing the duration of exposure to 10 d, medium with TDZ at (0.1, 0.4, and 1.0μM) also had significantly low vitrification of shoots. Medium containing (0.4 and 1.0μM), recorded low vitrification of shoots. However, the results did not significantly differ from those observed in medium containing higher levels of TDZ at 5.0μM. Prolonged exposures to TDZ for 24 and >40 d resulted in highly

vitrified shoots regardless of the level of TDZ used in the culture medium. Vitrification, which is characterized by watery and highly succulent appearance of shoots is thought to be a result of a disruption in osmotic potential, causing an increased water uptake by tissues (Pierik, 1987). The disruption in osmotic potential could be caused by nutrient salts in the medium, accumulation of PGRs in the medium, or the concentration of agar (Kyte, 190; Pierik, 1987; Preece, 1995; Ziv, 1991). Lu, 1993 reported hyperhydricity of shoots in woody plants regenerated *in-vitro*, and exposed to TDZ for a prolonged period. Prolonged exposures to TDZ could have resulted in accumulation of growth substances and other metabolites. TDZ induced elevation of endogenous growth substances (Hutchinson *et al.*, 1996a), could have resulted in high vitrification of shoots (Ziv, 1991). Similarly, Kyte (1990), reported vitrification in micro propagated shoots when cytokinin levels in the growth medium were high. Further evidence for the possible disruption of osmotic potential can be obtained from the fact that TDZ induce accumulation of mineral ions and other stress related metabolites (Murch *et al.*, 1997; Murch and saxena, 1997). Probably at high TDZ levels or prolonged exposures, a higher increase in mineral ions and other metabolites could have been released, which may have disrupted the osmotic potential, resulting to the vitrification of shoots. Again, we report for the first time the influence of concentration and duration of exposure to TDZ on the vitrification of shoots.

In conclusion, Vitrification increased with increasing duration of exposure to TDZ. Prolonged exposures resulted in highly vitrified shoots and low vitrification was achieved with shorter duration of exposure for 3 d at all levels of TDZ concentration, and 10 d at (0.1, 0.4, and 1.0μM TDZ).

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CONCLUSIONS AND RECOMMENDATIONS

TDZ is a promising growth regulator by promoting whole plant regeneration under *in-vitro* conditions. In the present study, TDZ stimulated morphogenetic responses with efficiency comparable to or greater than the conventionally used combination of auxin and cytokinin.

TDZ induced direct or indirect multiple and single shoot regeneration depending on the genotype and the level of concentration of TDZ used. Suggesting a possibility of shoot regeneration and the ability of TDZ to substitute for auxin and cytokinin requirement, to be genetically controlled at the molecular level. Indeed TDZ alone was found to be comparable to or more efficient in shoot regeneration than a combination of auxin and cytokinin. In addition, this study has helped elucidate morphogenic responses in plants previously not studied extensively. Further studies in ultrastructural analysis should be done to ascertain the regeneration pathways, in addition to using lower or higher concentrations of TDZ and type of auxins than those used in the present study. Besides, a better understanding of the physiological responses caused by TDZ in the selected ornamental plants was achieved. The ability to regenerate plants from shoot-tip explants with greater efficiency is expected to facilitate the multiplication of clean planting material and application of biotechnological tools. Furthermore, improved micropropagation procedures should be used to supplement and not to replace traditional vegetative propagation methods, which also need to be improved.

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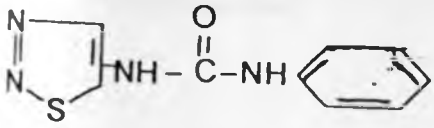
APPENDICES

A.1 Chemical structure of phenylureas (TDZ, CPPU), auxin (IAA), and Adenine-Type cytokinins (zeitin, kinetin and BA).



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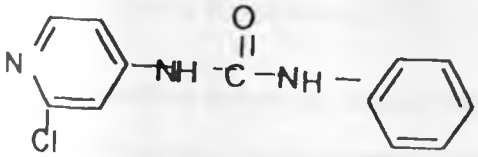

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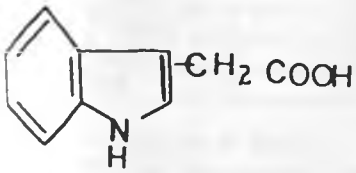

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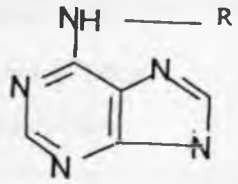

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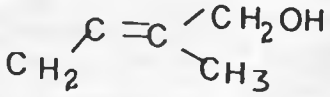

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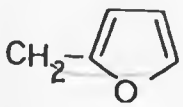
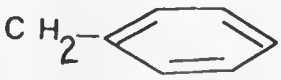


KINETIN



BA

A. 2 Physical and chemical properties of thidiazuron

Character	Description
Colour	Light yellow crystals
Oduor	Faint oduor
Trade name	DROPP
Chemical name	N-phenyl-N'-(1,2,3-thidiazol-5'yl) urea
Molecular formula	$C_9H_8N_4OS$
Molecular weight	220.2
Melting point	213°C
Storage	Dry conditions
Solubility (g/100ml)	Water: 0.002 Benzene: 0.0035 DMSO: >50.0

Source: Murthy et al., 1998.

A. 3 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean shoot length compared to a combination of BAP and NAA in tuberose shoot-tip cultures

Source of variation	df	Mean sum of squares					
		Mean shoot length (cm)					
		1week	3weeks	5weeks	8weeks	10weeks	12weeks
Treatment	13	0.035***	0.26***	0.74***	2.57***	4.7***	13.1***
Residual	42	0.002	0.03	0.02	0.01	0.05	0.16
Total	55						

df Degrees of freedom
*** Significant at 0.001 level of probability

A. 4 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean shoot quality rating compared to a combination of BAP and NAA in tuberose shoot-tip cultures

Source of variation	df	Mean sum of squares					
		Mean shoot quality rating					
		1week	3weeks	5weeks	8weeks	10weeks	12weeks
Treatment	13	0.36^{ns}	3.19^{***}	5.41^{***}	13.13^{***}	12.9^{***}	12.02^{***}
Residual	42	0.2	0.8	0.6	0.1	0.1	0.07
Total	55						

df Degrees of freedom
ns, *** nonsignificant and significant at 0.001 level of probability,respectively

A. 5 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean number of leaves compared to a combination of BAP and NAA in tuberose shoot-tip cultures

Source of variation	df	Mean sum of squares					
		Mean number of leaves					
		1week	3weeks	5weeks	8weeks	10weeks	12weeks
Treatment	13	0^{ns}	2.44^{***}	3.77^{***}	8.24^{***}	9.1^{***}	12.8^{***}
Residual	42	0	0.2	0.25	0.16	0.19	0.06
Total	55						

df Degrees of freedom
ns, *** nonsignificant and significant at 0.001 level of probability,respectively

A. 6 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean number of leaves compared to a combination of BAP and NAA in *Alstroemeria* shoot-tip cultures

Source of variation	df	Mean sum of squares							
		Weeks in culture							
		2weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	14 weeks	16 weeks
Treatment	8	0.71^{**}	3.23^{***}	6.97^{***}	14^{***}	20.1^{***}	19.4^{***}	14.7^{***}	17.6^{***}
Residual	27	27	0.19	0.19	0.2	0.4	0.4	0.5	0.3
Total	35								

df Degrees of freedom
, * Significant at 0.01 and 0.001 level of probability,respectively

A. 7 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean number of shoots compared to a combination of BAP and NAA in *Alstroemeria* shoot-tip cultures

Source of variation	df	Mean sum of squares							
		Weeks in culture							
		2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	14 weeks	16 weeks
Treatment	8	0.02 ns	0.84***	3.36***	6.1***	11***	13.8***	13.3***	17.8***
Residual	27	0.02	0.05	0.07	0.06	0.2	0.3	0.4	0.5
Total	35								

df Degrees of freedom
ns, *** nonsignificant and significant at 0.001 level of probability, respectively

A. 8 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean shoot quality rating compared to a combination of BAP and NAA in *Alstroemeria* shoot-tip cultures

Source of variation	df	Mean sum of squares							
		Weeks in culture							
		2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	14 weeks	16 weeks
Treatment	8	1.06***	1.46***	3.06***	4.06***	4.04***	3.73***	4.06***	4.5***
Residual	27	0.06	0.12	0.2	0.2	0.12	0.14	0.08	0.03
0.2Total	35								

df Degrees of freedom
*** Significant at 0.001 level of probability

A. 9 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean shoot length (cm) compared to a combination of BAP and NAA in *Alstroemeria* shoot-tip cultures

Source of variation	df	Mean sum of squares							
		Weeks in culture							
		2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	14 weeks	16 weeks
Treatment	8	1.05***	1.67***	2.44***	3.62***	4.39***	3.5***	3.53***	3.5***
Residual	27	0.01	0.02	0.02	0.06	0.06	0.12	0.09	0.08
Total	35								

df Degrees of freedom
*** Significant at 0.001 level of probability

A.10 ANOVA table for the effect of TDZ alone at various concentrations on the mean number of shoots compared to a combination of BAP and NAA in *Ornithogalum saundersiae* shoot-tip cultures

Source of variation	df	Mean sum of squares	
		Mean number of shoots	
		4 Weeks	10 weeks
Treatment	7	110.07***	673.64***
Residual	24	1.72	13
Total	31		

df Degrees of freedom
*** Significant at 0.001 level of probability

A.11 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean number of shoots compared to a combination of KIN and NAA in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares			
		Mean number of leaves			
		1week	2weeks	3weeks	4weeks
Treatment	8	0.06^{ns}	50***	440.1***	1035***
Residual	27	0.11	1.1	3.39	6.9
Total	35				

df Degrees of freedom
ns, *** nonsignificant and significant at 0.001 level of probability, respectively

A.12 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean shoot length (cm) compared to a combination of KIN and NAA in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares			
		Mean shoot quality rating			
		1week	2weeks	3weeks	4weeks
Treatment	8	0.31^{ns}	0.52^{ns}	0.61**	0.98**
Residual	27	0.2	0.37	0.17	0.2
Total	35				

df Degrees of freedom
ns, ** nonsignificant and significant at 0.01 level of probability, respectively

A.13 ANOVA table for the effect of TDZ alone at various concentration or in combination with NAA on the mean shoot quality rating compared to a combination of KIN and NAA in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares			
		Mean shoot length (cm)			
		1week	2weeks	3weeks	4weeks
Treatment	8	0.06ns	0.17ns	1.27***	1.43***
Residual	27	0.006	0.009	0.175	0.194
Total	35				

df Degrees of freedom
ns, *** nonsignificant and significant at 0.001 level of probability, respectively

A.14 ANOVA table for the effect of TDZ alone at various concentrations or in combination with NAA on the mean shoot vitrification rating compared to a combination of KIN and NAA in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares			
		Shoot Vitrification rating			
		1week	2weeks	3weeks	4weeks
Treatment	8	3.31***	3.0***	3.29***	3.19***
Residual	27	0.23	0.25	0.314	0.185
Total	35				

df Degrees of freedom
*** Significant at 0.001 level of probability

A.15 ANOVA table for the effect of dose and duration of exposure to TDZ on the mean number of shoots in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares
		Mean number of shoots
Level	3	1613.29***
Exposure	3	270.39***
Level*Exposure	9	66.81*
Residual	176	29.06
Total	191	

df Degrees of freedom
*, *** Significant at 0.05 and 0.001 level of probability, respectively

A.16 ANOVA table for the effect of dose and duration of exposure to TDZ on the mean shoot quality rating in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares
		Mean shoot quality rating
Level	3	13.125***
Exposure	3	10.069***
Level*Exposure	9	2.25***
Residual	176	0.42
Total	191	

df Degrees of freedom
*** Significant at 0.001 level of probability.

A.17 ANOVA table for the effect of dose and duration of exposure to TDZ on the mean shoot length (cm) in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares
		Mean shoot length (cm)
Level	3	0.748***
Exposure	3	0.380***
Level*Exposure	9	0.025 ns
Residual	176	0.014
Total	191	

df Degrees of freedom
ns, *** nonsignificant and significant at 0.001 level of probability, respectively

A.18 ANOVA table for the effect of dose and duration of exposure to TDZ on the vitrification rating of shoots in carnation shoot-tip cultures

Source of variation	df	Mean sum of squares
		Vitrification rating of shoots
Level	3	5.701***
Exposure	3	12.11***
Level*Exposure	9	1.11***
Residual	176	0.24
Total	191	

df Degrees of freedom
*** Significant at 0.001 level of probability