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DISSERTATION

IN VITRO PROPAGATION AND POLYPLOID INDUCTION OF KORARIMA (*Aframomum corrorima* (BRAUN) JANSEN) AND KRAWAN (*Amomum krervanh* PIERRE)

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Korarima and krawan are common spices and medicinal plants of economic importance. Shortage of clonal planting materials is a common problem encountered. The technique of micropropagation could be used to overcome this production problem. A study to determine the most suitable medium for *in vitro* multiplication of both species was conducted. *In vitro* germination of korarima seeds could easily be accomplished on distilled water soaked cotton pads. Of four media tested, supplemented with 5% coconut water, Murashige and Skoog (1962) (MS) medium was proved the best for culture initiation and subsequent proliferation of both plants. Rooting of both species was accomplished with a relative ease on the basal MS medium. The use of 0.5 mg l^{-1} thidiazuron (TDZ) in combination with 3 mg l^{-1} paclobutrazol (PBZ) exerted a strong synergistic effect upon shoot proliferation and growth of korarima. On the other hand, inclusion of PBZ in the culture medium had antagonistic effect on krawan cultures. Shoot multiplication was enhanced in both species through the simultaneous use of 0.5 mg l^{-1} TDZ and 2 mg l^{-1} imazalil (IMA). The medium added with 3 mg l^{-1} N^6-benzyladenine (BA) with 0.5 mg l^{-1} TDZ was also the best for shoot proliferation in korarima. However, krawan shoots responded best when the level of TDZ was increased to 0.75 mg l^{-1}. Friable creamy callus was best induced in korarima using 0.1 mg l^{-1} BA and 1 mg l^{-1} 2,4-D, with 1 g l^{-1} casein hydrolysate, while for krawan the level of 2,4-D needs to be raised to 4 mg l^{-1}. In both species, the calli did not regenerate on the different media tested. Doubling in chromosome number is believed to be beneficial for improvement of both species. The use of 10 µM oryzalin or 125 µM colchicine could produce solid tetraploids in korarima. An attempt in krawan produced only mixoploid.

Student's signature

Thesis Advisor's signature

19.04.04

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LIST OF ABBREVIATIONS

2,4-D	=	2,4-Dichlorophenoxy acetic acid
AC	=	Activated charcoal
ANOVA	=	Analysis of Variance
BA or BAP	=	N^6-benzyladenine or N^6-benzylaminopurine
BAK	=	3 mg l^{-1} BA with 1 mg l^{-1} kinetin
CBP	=	Cytokinin-binding protein
CH	=	Casein hydrolysate
CRD	=	Completely Randomized Design
CW	=	Coconut water
DMRT	=	Duncan's Multiple Range Test
DMSO	=	Dimethylsulfoxide
DW	=	Dry weight
EARO	=	Ethiopian Agricultural Research Organization
FW	=	Fresh weight
GLM	=	General linear model
GRI	=	Germination rate index
HMS	=	Half macro MS medium
HNMS	=	MS medium with half of its nitrate and ammonium ions
IMA	=	Imazalil (Fungaflor®)
IMABA-1	=	4 mg l^{-1} IMA with 0.1 mg l^{-1} BA
IMABA-2	=	2 mg l^{-1} IMA and 0.5 mg l^{-1} BA
IMATDZ	=	2 mg l^{-1} IMA and 0.5 mg l^{-1} thidiazuron (TDZ)
JARC	=	Jimma Agricultural Research Center
MDG	=	Mean days to germination
MS	=	Murashige and Skoog (1962) medium
NAA	=	1-naphtalene acetic acid
NH_4^+	=	Ammonium
NO_3^-	=	Nitrate
NSULA	=	Number of stomata per unit leaf area

LIST OF ABBREVIATIONS (cont'd)

PBZ	=	Paclobutrazol
PGR-free	=	Plant growth regulator-free
SH	=	Schenk and Hildebrandt (1972) medium
SNK	=	Student-Newman-Keuls' (SNK) mean separation test
TDZ	=	Thidiazuron (Dropp®)
v/v	=	volume by volume
w/v	=	weight by volume

IN VITRO PROPAGATION AND POLYPLOID INDUCTION OF KORARIMA (*Aframomum corrorima* (BRAUN) JANSEN) AND KRAWAN (*Amomum krervanh* PIERRE)

INTRODUCTION

Korarima (*Aframomum corrorima)* and krawan (*Amomum krervanh*) are sometimes also called the Ethiopian and Siam cardamom, respectively. They are close relatives of the widely known Indian cardamom (*Elettaria cardamomum* Maton). With regard to their origin, korarima is endemic to Ethiopia, while krawan is to Thailand and Cambodia (Purseglove *et al.*, 1981). In both species, the seeds are the economic parts of the plant, and are commonly used for flavoring foods and/or beverages and in traditional medicine. The seeds of korarima are part and parcel of the daily dishes of the Ethiopians (Jansen, 1981), while that of krawan are commonly used to flavor curries (Paisooksantivatana, 1996). In Indo-China, seeds of krawan are traded as a condiment and spice (Binh, 1999). With respect to their medicinal attributes, korarima seeds are used as carminative, purgative, as well as tonic (Jansen, 1981). Similarly, krawan fruits are used to treat indigestion, liver and uterus diseases, rheumatism, diarrhea and asthenic after dysentery, and as febrifuge, antiemetic and antitoxic of alcohol (Binh, 1999). It is one of the few Thai medicinal plants widely used in the traditional medicine and with a commercial potential (Subansenee, 1995).

With regard to their taste, Purseglove *et al.* (1981) and Katzer (1999) had described seeds of krawan to be very close in flavor to that of the Indian cardamom. Different reports state the oil composition of korarima to be qualitatively similar to that of cardamom, except for the reduced content of terpinyl acetate, which is the major component in the latter (Sebsebe, 1993). Yield and chemical composition shows considerable variation with clone in korarima. Therefore, it is essential to devise ways and means to rapidly propagate those identified promising lines, but there has not been any technology to enable this. In line with krawan, some exploration

works were undertaken to document the distribution of the crop in the country. Some efforts of germplasm collection and preservation were carried out and potential areas of production were identified (Paisooksantivatana, 1996). Besides, the medicinal attributes of the crop were also studied and encouraging results were recorded (Saralamp *et al.,* 1996). Preliminary studies conducted on krawan seed also revealed its essential oil content to be around 0.93% (Wasuwat, 1993).

Compared to cardamom, korarima has a relatively wider adaptation and ca 5.5-fold productivity. The current returns from korarima are much higher than most cereals grown in the region. Furthermore, as this yield is obtained from the wild crop, there is a lot of room for improvement with the use of selected clones and the adoption of improved cultural practices (Ethiopian Agricultural Research Organization, EARO, 2000). Krawan also has a relatively better adaptation and a nearly three- to six-fold productivity than that of cardamom (Chomchalow *et al.,* 2003). It can grow between 60 - 1300 meters above sea level (masl), though the best results are obtained from the higher altitudes (Paisooksantivatana, 1996). The shade trees used in the agricultural system of both crops are also a major source of fuel and timber, signifying additional income for the owners.

As reported by Jansen (1981) and Lock (1997), Ethiopia had been well known for its considerable exports of korarima seeds to Sudan, Egypt, Arabia, Iran, India and the Scandinavian markets. In the early 70's, an average of 118 and 11 tons of dried capsules were annually exported to Sweden and Finland, respectively. Likewise, the Southeast Asian countries were annually exporting about 400 - 1000 tones of krawan to the world market. Among these, Thailand was the major supplier. Both these exports of korarima and krawan were mainly meant to substitute cardamom, which is a renowned spice in the world since the ancient times (Purseglove *et al.,* 1981).

However, this encouraging trend of korarima and krawan export had greatly fluctuated during the past few decades. The total annual export of korarima had reduced to less than 60 tones in the years 1994 - 1998, fetching only 2.1 million USD

(Chanyalew, 1999). In the year 2001, there was a very serious shortage of korarima supply in the market and the price had reached as high as 23.5 USD/kg. On the other hand, as reviewed by Sinchaisri (1993), the export of krawan in the year 1991 from Thailand was only 8 tones with a value of 16 thousand USD. Other reports also attest a significant reduction in the value of Thailand's spices export (of which krawan is a part) from 2.14 million USD during 1989 to 1.42 million USD in the year 1993, with a drastic reduction of export quantity from 846 tons in the year 1990 to only 289 in the year 1993 (Subansenee, 1995).

The prevailing unstable korarima market situations could basically be ascribed to the reduction of production as a result of the ever-increasing destruction of the natural forests (EARO, 2000), due to the expansion of arable and grazing land, urbanization and bush fire. Krawan is also facing a serious threat of extinction due to devastation of the natural forests (Paisooksantivatana, 1996). Therefore, the current irreversible destruction of their natural habitat is even threatening the mere existence of both crops in these countries. Besides, there are no visible activities regarding establishment of new plantations.

A large quantity of propagation materials of the desired clone is needed for further expansion of production area in korarima and krawan. The use of vegetative materials comprising at least one mature and another young shoot (together with their rhizomes) is the common method of propagation of korarima (Purseglove *et al.,* 1981) and krawan (Paisooksantivatana, 1996). However, the technique has its own drawbacks, as it involves considerable sacrifice of potential productive plant stands. Therefore, the ongoing efforts directed towards the improvement of both crops should be substantiated with subsequent tasks, such as development of efficient techniques for rapid propagation of promising lines. Successful efforts had been made to develop *in vitro* propagation protocols for different plant species of the family Zingiberaceae, including cardamom, ginger and turmeric. But to our knowledge, no comprehensive work has yet been conducted on korarima and krawan.

In addition, plant tissue culture technology is considered as a potential means for creating genetic variability and bringing about significant changes in the chemical composition of plants. Especially, the technique of *in vitro* polyploid induction has resulted in considerable alterations of the chemical constituents, morphology and/or agronomic characters of different species, including cardamom (Sudharshan, 1989). To this end, different chemicals as well as techniques of polyploid induction had been reported in several herbaceous crop species, including members of the family Zingiberaceae (Hamill *et al.,* 1992; van Duren *et al.*, 1996; Adaniya and Shirai, 2001). However, no efforts were so far directed to use this technique for the improvement of either korarima or krawan.

Objectives

The objectives of this study are:

- To develop *in vitro* propagation protocols for korarima and krawan
- To assess the most effective techniques for *in vitro* polyploid induction for korarima and krawan

LITERATURE REVIEW

Taxonomy

Both korarima and krawan are aromatic perennial monocotyledonous herbs. They are close relatives of cardamom (*Elettaria cardamomum* Maton), which according to Uhl (2000) is also known as the "Queen of spices" and the world's third most expensive spice, after saffron and vanilla. According to Lock (1997), the family Zingiberaceae, to which both korarima and krawan belong, comprises about 50 genera and some 1300 species. Most of these plant species are found in Southeast Asia. Three genera and about 60 – 80 species are reported to be indigenous to Africa. However, *Aframomum* is the only genus of the African Zingiberaceae that has been cultivated (Lock, 1979). On the other hand; *Amomum* consists of some 100 species found distributed in East Asia, from India and China, throughout the Malayan peninsula, to tropical Australia (Binh, 1999). Among these, *Amomum krervanh*, *Amomum villosum*, *Amomum xanthioides* and *Amomum uliginosum*, are grown in Thailand for their medicinal values.

The leaf sheathes of korarima are long and superimposed to form the pseudostem, which reaches some 2 m in height (Jansen, 1981; Lock, 1997). The pseudostems of krawan are reported to reach up to 3 m (Paisooksantivatana, 1996). Korarima leaves are distichous and about 1 – 8 cm apart (Jansen, 1981; Lock, 1997), yellow–green in color and in most cases possess ciliated margins. They are about 10 – 30 X 2.5 – 6 cm in size, elliptic to oblong in shape, acuminate at the apex, leathery, and deeply grooved above. As reviewed by Dahlgren *et al.* (1985), the venation is indicated to be generally pinnate-parallel, with a prominent mid-vein, straight and parallel to arching secondary veins having dark green color. Korarima plants have strong fibrous underground scaly rhizomes. The rhizomes are about 6 X 4 cm and brown to red brown in color. Roots are borne on the surface layer of the rhizomes. They are up to 4 mm in diameter, fibrous in nature and have whitish to light brown color (Jansen, 1981; Lock, 1997).

The leaves of krawan are simple, alternate, exstipulate, and oblong with a size of 8 - 15 cm width and 40 - 60 cm length (Gagnepain, 1908). According to Chomchalow *et al.* (2003) the underground stem of krawan is a bulbous rhizome with 8 - 20 nodes. The rhizomes bear densely spreading roots just below the soil surface (Paisooksantivatana, 1996). In both species, new suckers are borne from their underground rhizomes to replace the old ones and to enlarge the clump.

The flowers of korarima are white to pale violet in color (Jansen, 1981). Its inflorescence are 4 to 6-flowered with short stalked head, arising singly or two together on a peduncle 3 – 8 cm long. The peduncle is usually curved, purplish brown, nearly ovate and covered with scales of ca 2.5 X 1.5 cm. It arises directly from the rhizome near the base of the leafy stem and sometimes at the end of the rhizomatous runner (Lock, 1978; Jansen, 1981; Lock, 1997). The flowering period of korarima extends between January and September in different locations, and harvesting commences when the fruits mature (i.e. after 2 – 3 months) (Jansen, 1981).

The flowers of krawan are white colored and in dense spikes (Gagnepain, 1908; Saralamp *et al.,* 1996; Binh, 1999). The inflorescences are cylindrical to conical, up to 11 cm long and arise directly from the rhizome (Binh, 1999). It flowers between December and January (cool season) and its harvesting period extends from July to August, and September to October for plants grown at the lower and higher altitudes, respectively (Paisooksantivatana, 1996).

As reported by Jansen (1981), the fruits of korarima are indehiscent, fleshy and sub-conical in shape. They are shiny-green at their early stages of development, bright red at maturity, while brown to gray-brown after drying. Each flask shaped dried fruit (ca. 3 – 7 cm long and 1.5 – 3.5 cm diameter) is characteristically trilocular, containing 45 – 65 seeds. The seeds are aromatic, about 2 X 3 mm in size, with shiny brown color, spherical shape, and covered with a thin mucilaginous layer

(Lock, 1997). The yield of korarima under the untended condition is ca 0.55 tones/ha (EARO, 2000).

According to Chomchalow *et al.* (2003), krawan fruit is a glabrous capsule, globose in shape and borne 10 - 20 per bunch. It has got 9 - 18 seeds per capsule and strongly scented, similar to camphor with a pungent taste. The capsules measure about 1.5 cm in diameter, round, globular, smooth, ribbed, and obscurely triangular having a brownish-white color (Binh, 1999). The yield of krawan is reported to be around 300 - 625 kg/ha (Chomchalow *et al.*, 2003).

Ecology and Distribution

Unlike most other species of the family Zingiberaceae, the distribution of korarima is restricted only to Ethiopia. The crop is found naturally growing in the tropical rain forest regions of Southern, South western and Western parts of Ethiopia. Although some people had hinted its existence on the Aloma plateau of the Sudan, most reports attest the species to be endemic to Ethiopia (Jansen, 1981; Lock, 1997). It grows wild in partially shaded areas with altitudinal ranges of 1350 – 2000 m, with rainfall of 1300 – 2000 mm or more and no distinct dry season (Goettsch, 1991; Sebsebe, 1993). It also requires an average temperature of ca 20° C (Westephal, 1975). However, this range could also be extended through cultivation, as it is evidenced from its current growth outside the tropical rain forest regions of the country (Lock, 1997). The natural habitat of the crop is characterized by heavy shades, where soil disturbances are absent, soil moisture and organic matter are abundant and the soil is loose and friable (Westephal, 1975).

Krawan is currently found growing wild under the forest cover in Thailand, Lao, Cambodia, Myanmar, Vietnam and South China (Binh, 1999). The crop grows in the damp places of the tropics and its natural habitat is stated to be on the mountain slopes and ravines under partial shade of the tropical evergreen forest vegetation (Paisooksantivatana, 1996; Saralamp *et al.*, 1996). As reviewed by Paisooksantivatana

(1996), the crop is found distributed from 15° 30' N in the North to 7° 0' N in the South of Thailand, within the altitudinal ranges of 60 - 1300 masl. The annual rainfall of these areas is between 1500 - 3500 mm, while their temperature ranges between 25° and 35°C. However, relatively higher yield is obtained from plants cultivated at the higher altitudes (i.e. 850 - 1300 masl). Krawan plants start flowering after 2 - 3 years of planting (Chomchalow *et al.*, 2003). Like that of korarima, krawan is also facing a serious problem of extinction, due to devastation of the natural forests (Paisooksantivatana, 1996).

Conventional Methods of Propagation

Both korarima and krawan can be propagated by sexual (using seed) and asexual means (by using the subterranean rhizome cuttings). However, both these conventional propagation methods do have their own drawbacks. Alike with that of cardamom (Purseglove, 1975), propagation of korarima from seeds is discouraged due to its delayed and erratic germination. Furthermore, korarima plants propagated from seeds require longer time (not less than 5 years) to complete their juvenile phase and reach maturity.

The use of vegetative materials involving at least one mature and another young shoot together with their rhizomes is the most common method of propagation in both krawan (Chomchalow *et al.*, 2003) and korarima (Purseglove *et al.*, 1981). As compared to the sexual, the asexual method enables the grower to successfully propagate selected clones maintaining true-to-typeness. Such vegetatively propagated plants are also praised for their provision of earlier and higher yields. Despite these positive aspects, the vegetative technique also has its own drawbacks. It involves a considerable sacrifice of potential productive plant stands (Purseglove *et al.*, 1981). The technique is also labeled as extremely dangerous whenever there is a threat of disease and/or pest incidence.

In Vitro Seed Germination

In vitro germination of seeds could be attained using different techniques. In most cases, agar-solidified basal salt media are stated effective for a number of species (Almaz *et al.*, 2000, Jordan *et al.*, 2001), while in others diluted salt concentrations are stated best (Uniyal and Ram, 1996, Bringmann *et al.*, 2002 Padilla and Encina, 2003). However, still others need no salt supplement in the medium for germination and they can successfully germinate on water agar or any other support (e.g. sterilized cotton pad, moist paper, etc.) rinsed with distilled water. Mineral demand during the process of germination depends on the amount of food reserves in the seed, which in turn is related to the species (Padilla and Encina, 2003).

Therefore, a PGR-free MS medium was used for germinating *Sophora toromiro* (Jordan *et al.*, 2001) and *Swainsona alsula* seeds (Yang *et al.*, 2001). However, Padilla and Encina (2003) indicated MS basal medium to be the worst for germination of *Annona cherimola* seeds.

Several reports indicate the best way to get effective *in vitro* germination is through the use of low salt media. Srinivasa Rao *et al.* (1982) used White (1963) (W or White's) medium for the *in vitro* germination of cardamom seeds. Medium with half diluted MS salts was also reported effective in *Bulbine caulescens* and two *Kniphofia* species (Bringmann *et al.*, 2002), as well as in *Dianthus zeyheri* (Crouch and van Staden, 1993). However, Uniyal and Ram (1996) had observed further dilution of the basal medium to $1/5^{th}$ to be more effective for the *in vitro* germination of *Dalzellia zeylanica.*

On the other hand, the use of distilled water (DW) or water agar was reported to be the best for *Annona muricata* (Lemos and Blake, 1996), *Sophora toromiro* (Jordan *et al.*, 2001), St. John's wort (*Hypericum perforatum* 'Anthos') (Murch *et al.*, 2000) and *Annona cherimola* (Padilla and Encina, 2003).

Micropropagation of Related Genera

With the advent of modern biotechnology, plant tissue culture or micropropagation is the best alternative to cope up with the problems from the conventional sexual and asexual propagation techniques. It also plays an important role in the manipulation of plants for improved agronomic performance (Smith and Drew, 1990). Successful efforts were made to micropropagate cardamom (*Elettaria cardamomum*) and large cardamom (*Amomum subulatum*), as well as several close relatives of ginger and turmeric. Lukose *et al.* (1993) reported considerable differences in some vegetative characters of micropropagated cardamom plants from those grown from suckers and/or seedlings. The overall performance and quality of micropropagated plants, especially the number of yielding tillers/plant, was also observed to be superior to those raised from suckers and seeds (Nirmal Babu *et al.*, 1998). Besides, Sudharshan and Bhat (1998) stated tissue cultured cardamom plants to be superior to seedlings grown from open pollinated mother plants, in their growth performance, yield and several quality attributes.

1. Selection of explant

Different types of explants were used for micropropagating several plant species of the family Zingiberaceae (Appendix Tables 1 - 12).

Cardamom and other close relatives

According to Reghunath and Bajaj (1992), the micropropagation studies of cardamom conducted so far could be summarized under three categories: i.e. callusing, adventitious bud formation and/or enhanced axillary branching. Therefore, different people had used different types of explant to attain their specific objectives.

For callus induction, Srinivasa Rao *et al.* (1982) used sprouts from cardamom seeds as a source of explant, but others (Reghunath, 1989; Reghunath and Bajaj,

1992; Bajaj *et al.*, 1993) used shoot buds (20 - 50 mm) pretreated with 200 mg l^{-1} kinetin or 6-benzylaminopurine (BA) *in vivo* for 6 - 24 hours.

With regard to clonal propagation, different people (Nadgauda *et al.*, 1983; Vatsya *et al.*, 1987; Nirmal Babu *et al.*, 1999) recommended young sprouted buds to be the best explants in cardamom. However, Kumar *et al.* (1985) succeeded in micropropagating this same species using panicle primordium (15 – 20 mm) as a source of explant. From their *in vitro* studies, Reghunath and Bajaj (1992) reported shoot premordium (20 – 50 mm long) as the best explant for cardamom micropropagation. According to Bajaj *et al.* (1993), shoot meristems (3 – 4 mm long) obtained from *in vitro* shoot proliferation cultures (pre-cultured on Murashige and Skoog (1962), MS, basal medium containing 1% activated charcoal for a week) were the best explants for clonal propagation of cardamom. Reghunath and Priyadarshan (1993) also succeeded in propagating this species using juvenile shoot primordia (20 - 30 mm long) obtained from *in vitro* cultured mother plants. On the other hand, Sajina and his associates (1997) were able to propagate large cardamom (*Amomum subulatum*) using rhizome buds as their source of explant.

Ginger and other close relatives

In ginger, Hosoki and Sagawa (1977) had used emerging buds from stored rhizomes for their micropropagation work. Buds were primarily trimmed to 2 – 3 mm before culture so as to minimize contamination. However, Nel (1985) used excised roots from ginger plantlets (obtained from shoot tip cultures as explants) for his *in vitro* multiplication studies in South Africa. On the other hand, Ilahi and Jabeen (1987) made use of different types of explants, i.e. stem cuttings from 3-month old plants, young buds, rhizome cuttings with shoot bud primordia and juvenile shoots for *in vitro* propagation. They were able to produce callus from juvenile shoots and young buds, but not on the stem explants. Inden *et al.* (1988) used excised shoot tips from stored ginger rhizomes for the production of adventitious shoot primordia. From their study involving cultured leafy aerial pseudostems and decapitated crown sections,

Ikeda and Tanabe (1989) recommended the use of high quality rhizome materials for ginger micropropagation. Others (Bhagyalakshmi and Singh, 1988) used meristems with or without leaf primordia to induce shoot regeneration in ginger, while Sakamura and Suga (1989) made use of shoot tip explants (0.3 mm) to attain this same purpose. Malamug *et al.* (1991) induced callus from shoot tip explants of ginger, while Nirmal Babu *et al.* (1992) used the inner most tissues of young leaves.

In another instance, Dekkers *et al.* (1991) successfully micropropagated ginger and some of its close relatives using different explants. They used dissected axillary buds from rhizomes in the cases of *Zingiber officinale* and lempoyang (*Zingiber* sp.), aerial stem nodes in *Costus afer* and *Costus lacanusianus*, while bulbils arising in the inflorescence in *Alpina purpurata* for their *in vitro* studies. However, Brain and Richard (1993) used inflorescence axis sections with the bract base and vegetative axillary bud for regenerating *Alpina purpurata*. In *Kaempferia galanga*, young sprouting buds (6 - 8 mm) excised from rhizome were effectively used as explants (Vincent *et al.,* 1991). In *Zingiber cassumunar*, Poungpet and Krisana (1993) made use of shoot tips dissected from rhizomes in storage to attain this same purpose. Likewise, axillary buds (5 - 6 mm long) from rhizomes and emerging buds from stored rhizomes were used for micropropagating *Zingiber spectabile* (Faria and Illg, 1995), and *Alpina galanga* (Borthakur *et al.*, 1999), respectively.

Turmeric and other close relatives

From their work of clonal propagation involving different species of *Curcuma*, Yasuda *et al.* (1988) reported the possibility of using stem tips, rhizome buds and rhizome slices (ca 1 mm) as source of explant.

Several people successfully induced callus from sprouting buds in different species of *Curcuma,* including *Curcuma longa* (Shetty *et al.,* 1982; Neera *et al.,* 1999), *Curcuma domestica* (Mukhri and Yamaguchi 1986; Yasuda *et al.*, 1988),

Curcuma xanthorhiza (Mukhri and Yamaguchi, 1986), as well as *Curcuma aromatica* and *Curcuma zedoaria* (Yasuda *et al.*, 1988).

With respect to clonal propagation, Nadgauda *et al.* (1978) recommended the use of stem tips of rhizome buds (≥ 2 – 5 mm) as a source of explant in turmeric. Likewise, several other workers (Kuruvinashetti and Iyer, 1982; Shetty *et al.*, 1982; Keshavachndran and Khader, 1989) also used a nearly identical protocol for the rapid multiplication of *Curcuma domestica,* which involved buds excised from sprouting rhizomes as a source of explant. De Winnaar (1989) also used emerging buds (5 – 10 mm) excised from stored rhizomes as an explant for micropropagating turmeric. These were hence dissected, washed, sterilized, and then trimmed to contain the meristems together with a few leaf primordia.

On the other hand, Yasuda *et al.* (1988) obtained successful regeneration of *Curcuma zedoaria* plantlets from stem tip explants. Balachandran *et al.* (1990) also reported the efficiency of excised rhizome buds for *in vitro* multiplication of different *Curcuma* spp., whereby shoot and roots were produced simultaneously within 4 weeks time. As reported by Sugaya (1992), the terminal and lateral buds, which were excised and trimmed in a pyramid form (1 mm height and 1 mm width at the base), were the best explants for propagating *Curcuma zedoaria*. Besides, he also stated stem tips excised from the rhizome to be the best explants for micropropagating *Curcuma domestica, Curcuma zedoaria* and *Curcuma aromatica.* In another instance, Wannakrairoj (1997) has successfully used both young inflorescence (at the stage where all their bracts were tightly close) and buds from field grown rhizomes as sources of explant in clonal propagation of patumma (*Curcuma alismatifolia*). Similarly, in their *in vitro* studies of turmeric, Salvi *et al.* (2001; 2002) effectively used bud sprouts obtained from rhizomes planted in the soil.

2. Explant disinfestation

Several techniques had been employed to disinfest explants of plant species in the family Zingiberaceae prior to *in vitro* culture.

Cardamom and other close relatives

Reghunath and Bajaj (1992) used 95% ethanol and 0.15% mercuric chloride to cleanse cardamom explants obtained from the field. Reghunath and Priyadarshan (1993) also surface disinfested their cardamom explants using 95% ethanol for 15 seconds, followed by a treatment with 0.1% mercuric chloride (added with one drop of Teepol/50 ml) for 1 – 2 minutes and twice rinsing with distilled water. Similarly, Nirmal Babu *et al.* (1999) washed their cardamom rhizome pieces with sprouting buds under running tap water and then immersed them in a 0.1% mercuric chloride solution for 5 – 10 minutes, followed by 3 – 5 times rinsing using distilled water. In the case of *Amomum subulatum,* Sajina *et al.* (1997) washed the young sprouting buds from rhizome using running tap water and treated them with 0.3% copper oxychloride and Teepol for 1 hr. Later, the explants were immersed in 0.1% mercuric chloride solution for 5 - 7 min for surface sterilization (Appendix Table 1).

Ginger and other close relatives

Different techniques were used for sterilizing explants of ginger and its close relatives (Appendix Table 2). Therefore, Hosoki and Sagawa (1977) surface sterilized slices of ginger rhizome using 10% Clorox (0.5% sodium hypochlorite) for 10 minutes followed by twice rinsing with sterilized water, though only half were successfully cleansed. Others (Bhagyalakshmi and Singh, 1988) disinfested their sprouted ginger rhizomes using 0.2% aqueous solution of mercuric chloride followed by several rinsing with distilled water. Balachandran *et al.* (1990) also cleansed sprouting buds of *Zingiber officinale* using a 0.1% solution of mercuric chloride for 15 minutes, followed by 5 - 6 rinsing with distilled water. Malamug *et al.* (1991)

disinfested the growing buds obtained from rhizomes by cleaning them under running water for five minutes, followed by washing in ultrasonic washer using a 0.1% (v/v) detergent solution (benzalkonium chloride: Osvan) for five minutes and rinsing with 70% ethanol. Then after, the cleansed buds were surface sterilized with sodium hypochlorite solution (0.5% active chlorine and a wetting agent, Tween-80) for 10 minutes followed by three times rinsing with sterile distilled water. In another instance, Nirmal Babu *et al.* (1992) initially washed ginger explants (aerial shoots composed of sheathing leaf bases enclosing the youngest leaf inside and cut into 4 – 5 cm long sections) in a running water then surface disinfected them with a 0.1% mercuric chloride solution for 5 – 10 minutes, followed by 3 - 5 times rinsing using distilled water.

In relation to some other related species of ginger, Dekkers *et al.* (1991) surface sterilized the dissected axillary buds of *Costus afer, Costus lacanusianus* and lempoyang using 1% potassium hypochlorite for 20 min, accompanied by twice rinsing with sterilized water. This same technique was used to surface sterilize aerial stem nodes of *Alpina purpurata.* In another case, Poungpet and Krisana (1993) immersed *Zingiber cassumunar* shoot tips (dissected from rhizomes in storage) in 95% ethanol for 1 minute and treated them with 30 mg l^{-1} of either chloramphenicol or cloxacilline or neomycin, or 15 mg l^{-1} amoxicillin. Later, the materials were surface-disinfested with 10% Clorox solution and 2 – 3 drops of Tween-20 for 30 minutes, and rinsed three times. Faria and Illg (1995) washed the excised axillary buds of *Zingiber spectabile* with running water for 5 min after some trimming. Subsequently, disinfestation was carried out using 2.5% aqueous solution of sodium hypochlorite, with one drop of Tween 80 per 100 ml (10% solution) and shaken for 20 min at 120 rpm. However, Borthakur *et al.* (1999) were able to disinfest emerging buds from stored rhizomes of *Alpina galanga* with 0.2% mercuric chloride for 5 – 7 minutes, which were primarily soaked in Tween-20 solution for 15 minutes, and finally rinsed three times.

Turmeric and other close relatives

According to Sato *et al.* (1987), buds of *Curcuma domestica* were surface sterilized by immersing in 75% ethanol for 30 seconds and consequent rinsing with distilled water. Later, the explants were put in 3% sodium hypochlorite solution for 10 minutes, followed by rinsing three times using distilled water. Balachandran *et al.* (1990) attained effective sterilization of *Curcuma domestica, Curcuma caesia and Curcuma aeruginosa* using 0.1% mercuric chloride solution for 15 minutes, and subsequent 5 – 6 times rinsing with distilled water. In the case of *Curcuma zedoaria,* this was realized by immersing the explants in 70% ethanol for 30 seconds, followed by treatment with 10% chlorinated lime and twice rinsing using distilled water (Sugaya, 1992). In turmeric, Salvi *et al.* (2001; 2002) disinfested their sprouting rhizomes collected from the field by rinsing with 70% ethyl alcohol for 30 seconds and subsequent sterilization using 0.1% mercuric chloride solution for 15 minutes, and 5 - 6 times washing with sterile water (Appendix Table 3).

As reported by Wannakrairoj (1997), patumma inflorescence were successfully sterilized by initial rinsing under running water for 30 min, followed by a few minute soaking in 70% ethanol. The explants were then transferred to 0.5% sodium hypochlorite solution and shaken for 15 min and finally rinsed with sterilized water for 3 min. In the case of field grown rhizomes of this same species, the collected rhizomes bearing axillary buds were allowed to dry for a week, then buds were excised and rinsed under running water for 30 min and later kept in hot water (52°C) for 5 - 10 min. Buds were then dipped in 70% ethanol, disinfested using 0.79% sodium hypochlorite for 10 min, and finally rinsed with sterilized water (Appendix Table 3).

3. Media composition

Nutrient media, including their mineral constituents together with qualitative and quantitative aspects of plant growth regulators play a decisive role in micropropagation (Vardja and Vardja, 2001). Different culture media compositions had been used for micropropagating plant species in the family Zingiberaceae as described in the following section.

3.1. Callus induction

Cardamom and other close relatives

Srinivasa Rao *et al.* (1982) conducted callus induction studies using seed sprouts of cardamom as explant. Thus, Murashige and Skoog (1962) (MS) medium together with 18% coconut water (CW) supplemented with 2 mg l^{-1} 2,4-D, either alone or in combination with 1 g l^{-1} casein hydrolysate and 0.5 mg l^{-1} BA was reported to be effective in inducing callus. Moreover, MS media added with 18% CW and 2 mg l^{-1} NAA, together with 1 mg l^{-1} IAA or IBA gave rise to callus that grew as vascular nodules, but medium with higher auxin concentration produced friable callus, which was not conducive for shoot bud induction (Appendix Table 4).

Others (Reghunath, 1989; Reghunath and Bajaj, 1992; Bajaj *et al.*, 1993) reported 5 g l^{-1} agar solidified MS medium supplemented with 4 mg l^{-1} NAA, 1 mg l^{-1} BAP and 5% CW to be the best for callus production from shoot primordium of cardamom. As described by Reghunath (1989), the calli produced from the aforementioned treatments were compact and semi-hard, which started morphogenesis when sub-cultured on an auxin free medium (i.e. containing 2 or 3 mg l^{-1} BAP together with 0.5 or 1 mg l^{-1} kinetin. However, media having 2,4-D produced profuse, white, friable callus that did not initiate shoot organogenesis (Appendix Table 4).

Ginger and other close relatives

According to Malamug *et al.* (1991), production of calli from ginger explants was realized by using MS major elements, Ringe and Nitsch (1968) (RN) minor elements and organic addenda, 2% sucrose and 0.8% agar, together with 2,4-D and BA at varied concentrations. However, the best results were obtained when shoot tips were cultured on the media supplemented with 1 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D, while high concentrations of 2,4-D and BA inhibited callus formation. This same medium also resulted in satisfactory growth of subcultures. In another instance, Nirmal Babu *et al.* (1992) successfully induced callus by culturing the inner most tissues of young ginger leaves on MS medium supplemented with 13.6 μM 2,4-D. In *Kaempferia galanga*, Vincent *et al.* (1991) used MS medium added with 1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP for callus induction from young sprouting rhizomes (Appendix Table 5).

Turmeric and other close relatives

In their work on *Curcuma longa*, Shetty *et al.* (1982) successfully initiated callus from sprouting buds using MS medium, having 40 g l^{-1} sucrose, supplemented with 0.2 mg l^{-1} kinetin, 0.4 mg l^{-1} BAP and 0.01 mg l^{-1} GA_3. In another instance, Mukhri and Yamaguchi (1986), produced callus by culturing emerging buds (2 - 3 mm) of *Curcuma domestica* from rhizomes on RN medium added with 2% sucrose and 0.8% agar, together with 10 mg l^{-1} BA and 1 mg l^{-1} 2,4-D, or using 10 mg l^{-1} BA and 15 mg l^{-1} NAA. However, in *Curcuma xanthorhiza* success was observed when 10 mg l^{-1} BA and 15 mg l^{-1} NAA were used in the medium. Yasuda *et al.* (1988) also cultured slices of *Curcuma domestica, Curcuma aromatica,* and *Curcuma zedoaria* rhizomes on MS medium supplemented with 1 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin to attain this same purpose. However, Sugaya (1992) recommended the use of MS or RN medium added with BA at 10 mg l^{-1} to promote callus formation in *Curcuma domestica.* Others (Neera *et al.,* 1999) also obtained maximum callus from buds of *Curcuma longa* cultured on MS media having BA and 2,4-D at the respective rates of either 1 mg l^{-1} and 0.5-1 mg l^{-1}, or 3 mg l^{-1} and 0.1 mg l^{-1}, or 5 mg l^{-1} and 0.1 mg l^{-1}. According

to Salvi *et al.* (2001), callus cultures in turmeric were initiated from leaf base explants cultured on MS medium added with 2 mg l^{-1} of dicamba, picloram or NAA in combination with 0.5 mg l^{-1} BA (Appendix Table 6).

3.2. Shoot induction and multiplication

Cardamom and other close relatives

Nadgauda *et al.* (1983) utilized MS medium supplemented with 0.5 mg l^{-1} BA, 0.5 mg l^{-1} kinetin, 2.0 mg l^{-1} IAA, 5% CW, 0.5 mg l^{-1} calcium pantothenate, and 0.1 mg l^{-1} biotin for regenerating cardamom. Under these conditions, shoots were observed to grow to over 40 mm within two months time. However, Kumar *et al.* (1985) developed cardamom plantlets directly from immature panicles using MS medium supplemented with NAA and kinetin (each at 0.5 mg l^{-1}), and BAP, calcium penthothenate and folic acid (0.1 mg l^{-1} each), together with 10% CW. On the other hand, Reghunath and Bajaj (1992) reported Schenk and Hildebrandt (1972) (SH) basal medium supplemented with 0.5 – 1 mg l^{-1} NAA, 3 mg l^{-1} kinetin or 3 – 4 mg l^{-1} BA and 12 – 15% CW as the best combination for multiplying cardamom from shoot bud primordium. In a latter report, however, Bajaj *et al.* (1993) stated MS medium added with 5% CW, 2 - 3 mg l^{-1} BA and 0.5 - 1 mg l^{-1} kinetin solidified with 5 g l^{-1} agar as the best formulation for clonal propagation of cardamom. According to Sajina *et al.* (1997), effective culture establishment was obtained in large cardamom by using MS medium containing only 3% sucrose and 0.5 mg l^{-1} kinetin. A modified MS medium supplemented with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} IBA was also reported to be the best for shoot regeneration in this same species (Appendix Table 7).

Ginger and other close relatives

Excised buds from stored rhizome were cultured on a modified MS medium with 2% sucrose, 0.8% agar, 1 mg l^{-1} BA and 1 mg l^{-1} NAA for clonal propagation in ginger. However, cultures devoid of NAA were observed to give best results (Hosoki

and Sagawa, 1977). Ilahi and Jabeen (1987) used ½-strength MS media supplemented with different combinations of growth regulators for culturing different types of ginger explants (stem cuttings, young buds, rhizome cuttings with shoot primordia and juvenile shoots). Thus, bud primordia were effectively developed from juvenile shoots of *Zingiber officinale* in the medium containing varying concentrations of 2,4-D and BA, which on further sub-culturing grew into plantlets (Appendix Table 8).

In their micropropagation study of ginger, Bhagyalakshmi and Singh (1988) initiated growth of sprouting buds using 0.8% agar solidified plant growth regulator free (PGR-free) MS medium. On the other hand, they made use of ¾-strength MS medium supplemented with 6% sucrose, 20% CW, 100 mg l^{-1} ascorbic acid (AA), 400 mg l^{-1} glutamine, 250 mg l^{-1} activated charcoal (AC), 0.5 mg l^{-1} BA, 0.4 mg l^{-1} IBA and 0.8% agar for culturing ginger meristems with only apical dome or together with one or two leaf primordia. Best multiplication of shoots was obtained from semi-solid ¾-strength MS medium containing 3% sucrose, 100 mg l^{-1} AA, 100 mg l^{-1} AC and 4 – 5 mg l^{-1} BA. They also reported liquid media to be less effective than solid media for ginger micropropagation. On the other hand, Inden *et al.* (1988) used a modified MS medium containing 5 mg l^{-1} BA and 0.5 mg l^{-1} NAA, together with 20 g l^{-1} sucrose and succeeded in shoot initiation and multiplication of ginger (Appendix Table 8).

According to Ikeda and Tanabe (1989), leaf aerial pseudostems cultured on solid MS medium with 11 μM BA and 0.6 μM NAA produced about five shoots. But, decapitated crown sections cultured in liquid MS media supplemented with 11 μM BA produced 10 shoots on the average. In another instance, Sakamura and Suga (1989) cultured their ginger explants on Gamborg *et al.* (1968) (B5 or Gamborg's) and MS media supplemented with NAA at five levels (0, 0.02, 0.2, 2, and 4 mg l^{-1}) and BAP at two levels (0.2 and 2 mg l^{-1}). Thus, B5 medium was reported to enhance induction of plantlets in ginger, while MS medium promoted branching of plantlet rhizomes. In this experiment, NAA and BAP each at the rate of 2 mg l^{-1} promoted production of multiple shoots. Dekkers *et al.* (1991) also cultured axillary buds of ginger excised from rhizomes on MS medium added with 44 μM BA and succeeded

in producing plantlets. On the other hand, Brain and Richard (1993) induced shoots from inflorescence axis sections of ginger (with bract base and vegetative axillary bud) cultured on agar solidified ½-MS basal salts and organics, added with 2% sucrose and 4.4 μM BAP (Appendix Table 8).

Kacker *et al.* (1993) also regenerated shoots from embryogenic callus of ginger on MS medium supplemented with 8.9 μM BA. While Sharma and Singh (1995) succeeded in obtaining shoot proliferation by culturing tissue culture regenerated shoots on MS medium added with 2 mg l^{-1} calcium pantothenate, 1 mg l^{-1} BAP, 0.05 mg l^{-1} NAA and 0.2 mg l^{-1} GA_3. Successful shoot multiplication was also obtained by culturing ca 0.5 cm actively growing buds of ginger explants on MS medium supplemented with 3% sucrose and 0.8% agar, together with 2 mg l^{-1} kinetin (Sharma and Singh, 1997). Likewise, Devi (1999) suggested MS medium supplemented with BAP and kinetin (each at 0.1 mg l^{-1}) to be the best for plantlet production from bud explants of ginger. Under such conditions transferring the plantlets to a medium containing 4 mg l^{-1} BAP was reported to promote shoot production (Appendix Table 8).

With regard to other close relatives of ginger, Dekkers *et al.* (1991) used MS medium added with 0.3 g l^{-1} AC and 4.4 μM BA to regenerate plantlets from aerial stem nodes of *Costus afer* and *Costus lacanusianus*. They used this same medium formula for plantlet regeneration in *Alpina purpurata* using bulbils arising on the inflorescence as a source of explant. According to Faria and Illg (1995), cultures of *Zingiber spectabile* were effectively initiated using MS salts (½-strength) added with 2 mg l^{-1} thiamin, 1 mg l^{-1} nicotinic acid, 2 mg l^{-1} pyridoxine, 100 mg l^{-1} *myo*-inositol and 500 mg l^{-1} CH, together with 20 g l^{-1} sucrose. The medium was gelled with 2 g l^{-1} gerlite and supplemented with 10 μM BA and 5 μM IAA or 5 μM NAA. In this same species, both shoot elongation and proliferation were successfully attained using ½-strength MS medium supplemented with 10 μM BA. Shoots of *Alpina purpurata* were successfully multiplied from inflorescence buds cultured on a similar basal medium as in the case of *Zingiber spectabile* above (Illg and Faria, 1995). In *Alpina galanga*,

Borthakur *et al.* (1999) initiated shoots using MS media, 3% sucrose and 0.8% agar, together with 10 mg l^{-1} thiamin (i.e. to replace the standard vitamins in MS), supplemented with 3.0 mg l^{-1} kinetin (Appendix Table 8).

Turmeric and other close relatives

Nadgauda *et al.* (1978) conducted micropropagation studies on turmeric using W, MS, as well as a modified MS medium, from which edomin-S' and auxin were omitted, while 10% CW, 0.1 mg l^{-1} kinetin and 0.2 mg l^{-1} BAP were supplemented. All the media were solidified using 0.8% agar. Of the different media types tested, a modified MS medium supplemented with 10% CW, 0.1 mg l^{-1} kinetin and 0.2 mg l^{-1} BAP gave good shoot regeneration. As reported by Kuruvinashetti and Iyer (1982), best shoot induction was observed in *Curcuma domestica* when buds excised from sprouting rhizomes were cultured on MS medium supplemented with 2 mg l^{-1} BAP and 1 mg l^{-1} kinetin (Appendix Table 9).

Shoot regeneration was also attained when calli from *Curcuma domestica* and *Curcuma xanthorhiza* were kept on agar solidified RN medium supplemented with 2% sucrose, together with 1 mg l^{-1} BA and 1 mg l^{-1} NAA. However, 1 mg l^{-1} BA was required for culturing stem tips (buds from rhizome) of *Curcuma domestica* explants on RN medium. On the other hand, RN medium added with 10 mg l^{-1} BA was reported effective to induce shoots from callus (Mukhri and Yamaguchi, 1986). Shoots and plantlets were successfully regenerated from stem tip explants of turmeric on MS medium supplemented with 1 mg l^{-1} kinetin and 1 mM phloroglusinol. But, MS medium added with 10 mg l^{-1} BA was reported to be effective for culture initiation from shoot explants (Sato *et al.*, 1987). Effective production of plantlets was also observed from stem tip (bud of rhizome) explants of *Curcuma domestica* and *Curcuma caesia* grown on agar solidified (0.8%) MS media added with 30 g l^{-1} sucrose and 3 mg l^{-1} BAP (Balachandran *et al.*, 1990) (Appendix Table 9).

According to Shetty *et al.* (1982), shoots of *Curcuma longa* were successfully regenerated using sprouting buds from stored rhizomes cultured on MS basal media together with 40 g l^{-1} sucrose, 0.2 mg l^{-1} kinetin, 0.4 mg l^{-1} BAP and 0.01 mg l^{-1} GA_3. Similarly, Keshavachandran and Khader (1989) also succeeded in regenerating plantlets from bud tissues of *Curcuma longa* grown on MS media having 4% sucrose, together with 1 mg l^{-1} kinetin and 1 mg l^{-1} BA. Recently, Salvi *et al.* (2001) reported effective regeneration (formation of green shoot primordia) of calli produced from leaf base explants of this same species, using MS medium solidified with 0.25% phytagel added with 5 mg l^{-1} BA and 0.1 mg l^{-1} TIBA or 0.1 mg l^{-1} 2,4-D. Furthermore, ½-strength MS medium together with 2% sucrose and 1 mg l^{-1} kinetin was reported effective in producing complete shoots from these regenerants. In *Curcuma longa,* buds collected from field grown rhizomes were successfully established and multiplied on semi-solid (0.8% agar) MS medium added with B5 vitamins and 30 g l^{-1} sucrose. For further multiplication and maintenance of the shoots MS basal medium added with 5 μM BA and 1 μM NAA, and solidified with 0.4% agar was reported effective (Salvi *et al.*, 2002) (Appendix Table 9).

On the other hand, plantlets of *Curcuma aeruginosa* were successfully regenerated from stem tip (bud of rhizome) explants cultured on MS medium added with 30 g l^{-1} sucrose and 0.8% agar, as well as 3.0 mg l^{-1} BAP (Balachandran *et al.*, 1990). However, Dekkers *et al.* (1991) used MS medium supplemented with 4.4 μM BA to attain this same objective in *Curcuma aeruginosa* and *Curcuma amada*. In other cases, Yasuda *et al.* (1988) reported their success in plantlet production from stem tip explants of *Curcuma zedoaria* using MS basal media added with 0 – 1 mg l^{-1} NAA or 0 – 3 mg l^{-1} BA. Wannakrairoj (1997) has succeeded in multiplying shoots from young inflorescence and axillary bud explants of *Curcuma alismatifolia* by using 13.32 μM l^{-1} BA supplemented MS medium added with 3% sucrose. On the other hand, according to Tyagi *et al.* (1998), proliferation of axillary buds was realized in *Curcuma longa, Curcuma aeruginosa* and *Curcuma caesia* by culturing rhizome buds on MS medium supplemented with 3 mg l^{-1} BAP (Appendix Table 9).

3.3. Root induction

Cardamom and other close relatives

To attain effective rooting, Nadgauda *et al.* (1983) used White's liquid medium for regenerated shoot tip explants of cardamom that were initially cultured on a modified MS medium. Reghunath and Bajaj (1992) obtained successful rooting by culturing excised axillary shoots of cardamom on a semi-solid MS medium (½-strength) with 0.5 – 1% AC for one-week, followed by subsequent transfer to liquid ½-strength MS medium, containing 1 – 1.5 mg l^{-1} IBA. This technique was also refined and successfully used by Bajaj *et al.* (1993) at a later time during their studies of cardamom micropropagation. On the other hand, root regeneration of large cardamom was reported to be best on MS medium supplemented with 1.0 mg l^{-1} BA and/or 0.5 mg l^{-1} IBA (Sajina *et al.*, 1997) (Appendix Table 10).

Ginger and other close relatives

Rooting in ginger was effected when excised shoot tips from stored rhizome were cultured on a modified MS medium added with 20 g l^{-1} sucrose, 5 mg l^{-1} BA and 0.5 mg l^{-1} NAA (Inden *et al.*, 1988). As reported by Ikeda and Tanabe (1989), leaf aerial pseudostems of ginger cultured on solid MS medium with 11 μM BA and 0.6 μM NAA produced about 15.3 roots; while better results (16.3 roots) were obtained when decapitated crown sections were grown in a liquid MS medium supplemented only with 11 μM BA. On the other hand, Sakamura and Suga (1989) reported lower levels of NAA to promote root growth in the presence of low BAP levels, while increased levels of the former (> 0.2 mg l^{-1}) induced only root growth without shoot (Appendix Table 11).

Ginger explants cultured on MS major elements, RN minor elements and organic addenda, supplemented with 2% sucrose, 0.8% agar, as well as 2,4-D and BA at varied concentrations, were observed to form roots, despite the presence of BA in

the medium (though they were of the root-hair type) (Malamug *et al.*, 1991). In the course of this study, well-developed roots were observed only on cultures with regenerated shoots, whereby those on the medium containing 1 mg l^{-1} NAA and 5 mg l^{-1} BA produced functional roots. However, Nirmal Babu *et al.* (1992) attained rooting in their ginger cultures using a liquid MS medium supplemented with 5.4 μM NAA. But, Poungpet and Krisana (1993) used 10 g l^{-1} agar solidified Linsmaier and Skoog (1965), LS, medium added with 20 g l^{-1} sucrose, 20 g l^{-1} AC and 1 mg l^{-1} BAP to attain this same objective. Brain and Richard (1993) successfully induced roots on their *in vitro* regenerated ginger shoots using agar solidified ½-strength MS salts and organics, added with 2% sucrose, but without any growth regulator. Similarly, Devi (1999) reported successful *in vitro* rooting of ginger using plant growth regulator-free MS medium (Appendix Table 11).

Turmeric and other close relatives

Rooting was attained in *Curcuma domestica* using White's medium added with Na_2MoO_4 and $CuCl_2$ each at 0.25 mg l^{-1} (Nadgauda *et al.*, 1978). In other instance, Kuruvinashetti and Iyer (1982) were able to induce roots in this same species using ½-strength MS liquid minerals together with 20 g l^{-1} sucrose. However, Mukhri and Yamaguchi (1986) were able to produce roots in *Curcuma domestica* and *Curcuma xanthorhiza* from calli cultured on a modified RN medium added with 2% sucrose, 0.8% agar, as well as BA and NAA each at 1 mg l^{-1}. However, this same objective was realized in *Curcuma domestica* using emerging buds (2 - 3 mm) from rhizomes cultured on a RN medium supplemented with 10 mg l^{-1} BA and 15 mg l^{-1} NAA. On the other hand, when stem tip explants were used, a semi-solid RN medium added with sucrose and 1 mg l^{-1} BA was reported effective for rooting. Shoots of turmeric were also reported to produce healthy roots on a ½-MS medium supplemented with 0.1 mg l^{-1} NAA (Sato *et al.*, 1987) (Appendix Table 12).

As reported by Keshavachandran and Khader (1989), rooting was observed in *Curcuma domestica* when explants of young sprouting buds excised from rhizome

were cultured on a modified MS medium having 40 g l^{-1} sucrose and 7 g l^{-1} agar, together with 1 mg l^{-1} BAP. On the other hand, de Winnaar (1989) attained root development by transferring their turmeric culture to a medium containing 1 mg l^{-1} BA and 2% sucrose. In contrast to all these reports, regenerated shoots from *Curcuma longa* calli produced roots and formed complete plantlets on PGR- free ½-strength MS basal medium (Salvi *et al.*, 2001) (Appendix Table 12).

4. Some potential plant growth regulators for *in vitro* shoot proliferation

Development of an effective micropropagation protocol requires, among others, assessing for potent plant growth regulators that could provide high rates of shoot production. To enable growth and differentiation, as well as stimulate multiple shoot or bud formation it is essential to culture explants on medium supplemented with relatively high levels of cytokinins (Mok *et al.*, 1987; Malik and Saxena, 1992a; Mok *et al.*, 2000; Inoue *et al.*, 2001). This is mainly due to the presence of cytokinin-degrading enzymes, cytokinin oxidases that cleave unsaturated N^6–isoprenoid side chains. However, the use of cytokinins at higher concentrations could also be so toxic to the plants and/or uneconomical. To cope up with such problems, different chemicals with relatively higher potentials were identified and recommended in the past few decades.

4.1. Benzylaminoadenine

Different cytokinins show varied degrees of activities in affecting axillary shoot formation *in vitro* (Preece *et al.*, 1991; Nielson *et al.*, 1993). Both natural and synthetic cytokinins are used in plant tissue culture. Naturally occurring cytokinins are a large group of structurally related compounds. According to Ska *et al.* (2001), the major naturally occurring cytokinins in plants are derivatives of isopentenyladenine and cytokinins with a hydroxylated side-chain. Of these naturally occurring cytokinins, zeatin and 2iP are used in plant tissue culture media.

However, their use is not widespread as they are expensive (particularly zeatin), relatively unstable and highly liable to degradation by cytokinin oxidases. Therefore, high concentrations of adenine-type cytokinins are usually needed for growth and differentiation of tissue cultures. On the other hand, the synthetic cytokinins, such as kinetin and BA, are stated less susceptible to these degradative enzymes but they are generally less active than the naturally occurring cytokinins (Mok *et al.,* 1987; Hare and van Staden, 1994b).

In general, the number of axillary shoots induced by endogenous cytokinins produced from explants grown on a medium without cytokinin is small. This is since only endogenous adenine-type cytokinins will bind to cytokinin-binding protein (CBP). Exogenously supplied cytokinin, such as BA leads to an elevated cytokinin effect, which could be ascribed to the occupation of more adenine-cytokinin sites on CBP (Nielsen *et al.*, 1995).

As compared to kinetin, BA was stated more effective for shoot proliferation in *Plumbago zeylanica* at optimal concentrations (Rout *et al.*, 1999). In another instance, BA was reported as the best hormone for shoot regeneration of the salt marsh grass, *Spartina alterniflora*, than TDZ, as the latter inhibited root formation in the subsequent root regeneration medium (Wang *et al.*, 2003). Nielson *et al.* (1993) also stated BA to be superior in efficacy than other cytokinins, including TDZ for shoot formation in *Miscanthus sinensis.* Therefore, BA and TDZ were reported to have similar effects in axillary shoot formation, when used at equal concentrations, though BA induced a significantly higher number of shoots than TDZ. In the aromatic and medicinal shrub *Vitex negundo*, 2.0 mg l^{-1} BA was most effective in inducing bud break than either kinetin or TDZ (Sahoo and Chand, 1998).

Of four cytokinins (BA, TDZ, kinetin and 2iP) studied in *Bacopa monniera* culture, the first two showed superior performance (Tiwari *et al.*, 2001). According to Salvi *et al.* (2000), BA induced high frequency of regeneration than TDZ in inflorescence cultures of turmeric, though the average number of shoots/explant was

relatively low. From his work on minor millet (*Paspalum scrobiculatum* L.), Rashid (2002) also stated BA as a highly potent cytokinin with respect to shoot induction. Similarly, Sinha *et al.* (2000) also reported the superiority of BA to TDZ in inducing shoot buds in *Albizia chinensis* culture. Malik and Saxena (1992a) stated BA as an indispensable cytokinin all along the entire period of culture for induction and further development of shoot buds in *Phaseolus vulgaris* L.

According to Huetteman and Preece (1993), addition of a second cytokinin to the proliferation medium could enhance shoot proliferation. Media formulations containing two different cytokinins could alter the number and quality of shoots produced, unlike cultures with a medium with only one cytokinin (Nielsen *et al.*, 1995). Therefore, BA could be used more effectively in combination with other cytokinins or other growth regulating substances. To this end, the shoot inducing effect of BA was strongly enhanced by the use of the imadizol fungicide, imazalil (Werbrouck and Debergh, 1995; 1996). Similar effects were also reported when BA was used together with other imadizol fungicides (prochloraz and triflumizole), or the growth retardant paclobutrazol in cultures of *Spathiphyllum floribundum* 'Petite' Schott (Werbrouck and Debergh, 1996; 1997).

On the other hand, BA provided a relatively better shoot elongation than other potent cytokinins, like TDZ (Babaoglu and Yorgancilar, 2000, Rai, 2002). This also makes BA a better substitute for TDZ and other highly potent cytokinins, in cases where shoot elongation is equally important as shoot proliferation.

4.2. Thidiazuron

Based on their chemical structure, cytokinins could be classified into at least two broad groups, the adenine and phenylurea derivatives. Both adenine and a side chain at the N^6 position are the common features of the naturally occurring cytokinins, as well as many synthetic ones (Mok *et al.*, 1987). However, since the past twenty years, a different group of compounds, N,N^1-diphenylurea (DPU) and other

phenylurea derivatives were reported to posses growth-promoting properties (Mok *et al.*, 1982; Capelle *et al.*, 1983; Thomas and Katterman, 1986; Fellman *et al.*, 1987; Huetteman and Preece, 1993).

These cytokinin-active phenylureas, including thidiazuron, TDZ, (DROPP®, *N*-phenyl=*N*'-(1,2,3-thidiazol-5'yl) urea), were reported to display biological properties that are qualitatively similar to the adenine-type cytokinins (Yip and Yang, 1986; Mok *et al.*, 1987; Huetteman and Preece, 1993). The phenylureas were also observed to stimulate *in vitro* meristem and shoot formation at very low concentrations (Fellman *et al.*, 1987). Beside induction of organogenesis, the chemicals exhibit the capacity to promote callus growth and stimulate ethylene production. Some of these phenylureas were hence stated to be useful and economical for tissue culture applications due to their high activities at comparatively lower rates (Mok *et al.*, 1987)

Among the different phenyl urea derivatives so far studied for use in plant tissue culture, TDZ is believed to be the most potent (Mok *et al.*, 1982; Huetteman and Preece, 1993; Murthy *et al.*, 1998). According to Murthy *et al.* (1998), it exhibits the unique property of mimicking the effects of both auxin and cytokinin in growth and differentiation of cultured plants despite its structural difference with auxins or purine-based cytokinins. It induces organogenesis at much lower concentrations by reducing the apical dominance and hence resulting in the production of adventitious and/or axillary buds on the cultured shoot tip explants (Huetteman and Preece, 1993). Therefore, in recent years, this particular phenyl urea derivative compound has especially emerged as a highly effective plant growth regulator in tissue culture systems of several woody, as well as dicotyledonous and herbaceous plant species (Murthy *et al.*, 1998).

The biological activity of TDZ was reported to be higher than or comparable to that of the most active adenine-type cytokinins at unusually low concentrations (Thomas and Katterman, 1986; Mok *et al.*, 1987; Malik and Saxena, 1992a; Kim *et*

al., 1997; Victor *et al.*, 1999b; Rai, 2002). Besides, unlike the adenine-type cytokinins, exposure of plant tissues to TDZ for a relatively short period is stated to be sufficient to stimulate regeneration (Hutchinson and Saxena, 1996a).

The role of TDZ in morphogenesis is closely associated with the metabolism of endogenous growth regulators (Murthy *et al.*, 1998; Murch and Saxena, 2001). TDZ affects the endogenous cytokinin metabolism and transforms tissues from cytokinin dependency to cytokinin autonomy (Capelle *et al.*, 1983). As reported by several workers (Thomas and Katterman, 1986; Murthy *et al.*, 1995; Hutchinson and Saxena, 1996b), this enhancement was stated to be due to the capacity of TDZ to stimulate endogenous cytokinin biosynthesis or alter endogenous cytokinin metabolism, resulting in an increased levels of the natural cytokinins in the plant system (Mok *et al.*, 1987). This was reported to be specifically associated with several factors, including an increase in the synthesis, a decrease in catabolism (Capelle *et al.*, 1983), or the release of biologically active cytokinin molecules from stored non-active forms (Murthy *et al.*, 1998).

Cytokinin oxidase is the only enzyme known to cause irreversible inactivation of cytokinins in plants. It cleaves unsaturated N^6 –isoprenoid side chains (Mok *et al.*, 1987; Murthy *et al.*, 1998; Galuszka *et al.*, 2000). However, the synthetic cytokinins, such as kinetin and BA, are reported to be less susceptible to this effect of cytokinin oxidase than the natural ones. But, they are required at higher concentrations to promote growth and differentiation of tissue cultures. On the other hand, TDZ and other phenyl urea derivatives are resistant to the oxidases, stable, and biologically active at lower concentrations, since they can effectively inhibit the activity of cytokinin oxidase and hence prevent breakdown of purines (Chatfield and Armstrong, 1986; Hare and van Staden, 1994a; 1994b).

According to Murthy *et al.* (1998), stunted growth, dark colored leaves, swollen green cotyledons, and reduced primary root growth with few secondary roots were common characteristics of plants cultured on TDZ supplemented media.

Suppression of shoot elongation due to the use of TDZ was reported in different plant species (Chand *et al.*, 1999; Fratini and Ruiz, 2002; Roussos and Pontikis, 2002). According to Thomas and Katterman (1986), these responses of cultured tissues could be related to the suppression of cytokinin breakdown and/or the continued biosynthesis of purine cytokinins. They are also associated with the resistance of TDZ to cytokinin oxidase or accumulation of endogenous cytokinin (Murthy *et al.*, 1998; Arinaitwe *et al.*, 2000). Others (Arinaitwe *et al.*, 2000; Karam and Al-Majathoub, 2000) ascribed the phenomena to the higher cytokinin activity of TDZ even at a lower concentration. However, the observed shoot stunting effect was stated as a temporary phenomenon, which can be rectified by subsequent transfer of the shoots to BA containing or PGR free media (Chand *et al.*, 1999; Rai, 2002).

As reported by Rashid (2002), unlike in dicots, only few studies were so far conducted in monocots involving TDZ. Therefore, the morpho-regulatory effect of this potent compound requires further investigation in monocotyledonous plant species. To this end, no work had so far been reported especially on plant species of the family Zingiberaceae, except the works of Salvi *et al.* (2000) and Prathanturarug *et al.* (2003) that stated successful induction of high average number of shoot buds per inflorescence explant of turmeric using TDZ.

4.3. Imazalil

On the other hand, the imadizol fungicide, imazalil (Fungaflor®, 1-ß-allyloxy-2,-4-dichlorophenthyl) imidazole), significantly enhanced the shoot inducing effect of BA in *Spathiphyllum floribundum* culture (Werbrouck and Debergh, 1996). In this experiment, imazalil (IMA) considerably enhanced the shoot-inducing effect of BA and this synergistic effect of imazalil was confirmed for cytokinins of different groups such as Z, mT, and TDZ. Therefore, the number of shoots produced from BA supplemented medium showed a positive correlation with IMA concentrations used in the study, and their size was finally reduced to a small meristematic dome. But IMA didn't reveal any cytokinin effect on medium devoid of exogenous cytokinins

(Werbrouck and Debergh, 1995; 1996). However, these synergistic effects of IMA with BA were observed to be species specific, in that all monocot species studied didn't respond positively to its inclusion in the culture media (Werbrouck and Debergh, 1995).

According to Werbrouck and Debergh (1996), this interaction of IMA with different cytokinin groups indicates its probable effect on the general mechanism of cytokinin action. Werbrouck and Debergh (1995) also reported its inhibitory effect on the catabolic enzymes. Besides, from a latest study on *Spathiphyllum floribundum*, inhibition of the normal induction of new shoots by BA through addition of GA_3 was also observed that suggested the effects of IMA to be partially associated with inhibition of GA biosynthesis (Werbrouck and Debergh, 1997). Here, addition of IMA was stated to stop GA formation and consequently enhanced shoot proliferation. Thus, this suppression of GA biosynthesis together with alteration of endogenous cytokinin metabolism could have resulted in the synergistic effects IMA with other cytokinins.

4.4. Paclobutrazol

Triazoles belong to a class of compounds known as ergosterol biosynthesis inhibitors. Some of these azole compounds interfere with gibberellin biosynthesis, together with secondary modulation of ABA, ethylene, cytokinins, and polyamines and hence influence plant morphogenesis, which indicates their possible role as plant growth regulators. Hence several triazole derivatives were developed and promoted as either fungicides or plant growth regulators worldwide (Fletcher *et al.*, 2000). The commercial triazole products are much more effective than many other plant growth regulators at a very lower rate (Fletcher *et al.*, 1986; Jung *et al.*, 1986; Davis *et al.*, 1988; Gilley and Fletcher, 1997).

Of the different triazole derivatives, paclobutrazol (2RS,3RS)-1(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol) was reported to be

much more effective in retarding growth (Davis *et al.*, 1988). Triazoles are generally known to inhibit GA biosynthesis. The modulation of GA level leads to subsequent events, which include reduction of plant height, a higher root to shoot ratio, and modified leaf morphology, as well as enhanced synthesis of photosynthetic pigments (Fletcher *et al.*, 2000; Senaratna *et al.*, 2002). As reviewed by Davis *et al.* (1988), the most common plant growth response to triazole treatment is reduced stem elongation and hence reduced height, which is caused due to a reduction in the internode length. It also reduces stem weight and leaf area. In some species, however, the effects of triazoles were minimal on shoot number, while in others (e.g. apple, peach) the chemicals resulted in substantial reduction.

Paclobutrazol (PBZ) synergistically enhanced the shoot inducing effect of BA in *Spathiphyllum floribundum* culture. However, PBZ alone didn't show any cytokinin effect in the absence of exogenously applied cytokinins (Werbrouck and Debergh, 1996). Similarly, addition of 1.0 mg l^{-1} PBZ to TDZ (0.2 mg l^{-1}) resulted in much more proliferation of banana buds as compared to the medium containing only TDZ, though it suppressed shoot elongation. The dwarf bud clusters induced on the medium added with TDZ and PBZ produced normal shoots after one to two subcultures on a medium supplemented with BA alone (Lee, 2001).

As reviewed by Davis *et al.* (1988), triazoles had several morphological effects on leaves. Similar to most other growth retardants, treated plants typically exhibit darker green color than untreated controls. These color variations in most cases had been correlated with the increase in chlorophyll content. Triazoles also reduce leaf area, but increase epicuticular wax, width, and thickness, together with increase in leaf dry weight per unit area (Davis *et al.*, 1988; Fletcher *et al.*, 2000). However, the effect of triazole treatment on production of new leaves depends upon the rate of application; in such a way that leaf production was observed to reduce at higher rates, while no changes were observed at lower rates (Braun and Garth, 1986).

The available reports on the effect of triazoles on root growth had been somewhat contradictory; but in most cases reveal reductions in growth (Davis *et al.*, 1988; Fletcher *et al.*, 2000). Triazole-treated plants mostly produce numerous thickened, fleshy roots, which could be associated with increased root diameter and decreased root length (Bausher and Yelenosky, 1986). Despite these, however, a higher root-to-shoot ratio is the common characteristic of triazole-treated plants, which primarily could be due to the drastic reduction in shoot growth (Davis *et al.*, 1988; Fletcher *et al.*, 2000). This could again be attributed to imbalanced photosynthate partitioning, which results in a relative inhibition of shoot growth compared to root growth (Davis *et al.*, 1988).

With respect to their metabolic fate, though the subject has not been well studied, most triazoles show a high chemical stability and thus tend to be catabolized at a very slow rate within the plant system. As summarized by Fletcher *et al.* (2000), it could be stated that all these and similar other effects induced by triazoles could be ascribed to the increased cytokinin levels in the plant system.

5. Culture conditions

Different people suggested several options as optimal culture condition for the species under the family Zingiberaceae. In line with cardamom, Nadgauda *et al.* (1983) used a modified liquid MS medium on a shaker under continuous light (100 lx) at 28°C for both shoot multiplication and root induction. In other cases, Vatsya *et al.* (1987) incubated cardamom explants at 25°C under 40 µmoles $/m^{-2}s^{-1}$ illumination using white fluorescent tubes for 12:12 hours. However, Reghunath and Bajaj (1992) reported temperature ranges of 21 – 25°C and 16 h photoperiod (2000 – 3000 lx) to be the best for cardamom micropropagation. In another instance, Bajaj *et al.* (1993) indicated temperatures of 23 ± 2°C and 19 h photoperiod (500 - 1000 lx) to be optimal for culturing cardamom explants. In large cardamom, culture establishment and multiplication were attained by adjusting pH of the medium to 5.8 (prior to

autoclaving) and using culture condition of 22 ± 2°C temperature and 12 h photoperiod of 2500 lx (Sajina *et al.*, 1997).

According to Hosoki and Sagawa (1977), medium pH 6.0, temperature of 27 ± 2°C and continuous light (2100 lx) incubation were essential to obtain best results in ginger clonal propagation. Devi (1999), however, stated culture conditions with pH 5.7 and temperature between 25 – 30°C, together with a 16 h photoperiod as optimal for ginger micropropagation. Inden *et al.* (1988) used pH of 5.8 and temperature of 25 ± 1°C with 16 h light under a light intensity of 3000 lx for culturing their explants. In another instance, Malamug *et al.* (1991) used temperature of 26 ± 1°C with 14 h photoperiods (cool white fluorescent light, 60 μmol $m^{-2}s^{-1}$) for sub-culturing regenerated plantlets of ginger. Kacker *et al.* (1993) cultured their ginger explants in the dark for callus induction and maintenance using pH 5.8 and temperature of 25 ± 2°C. However, they kept their cultures under 16 h photoperiod at 35 $\mu mol.m^{-2}s^{-1}$ for plantlet regeneration. Sharma and Singh (1997), on the other hand, used pH 5.8, temperature of 25 ± 1°C, together with 16 h light under 140 - 145 $\mu Em^{-2}s^{-1}$ light intensity for shoot multiplication in ginger.

As reported by Nadgauda *et al.* (1978) the optimal conditions for regeneration of turmeric were stated as a pH of 5.8 and daily incubation of the explants at 28°C for 12 h photoperiod (1000 lx) and at 25°C for the rest 12 h in the dark. However, Shetty *et al.* (1982) used culture conditions with pH 5.6 and temperature of 26 ± 1°C, as well as 12 h light from 40 watt fluorescent tubes. In other cases, Mukhri and Yamaguchi (1986) initially cultured buds of *Curcuma domestica* and *Curcuma xanthorhiza* at pH 6.0 and temperature of 25 ± 1°C in the dark. To stimulate shoot formation, root or embryoid cultures were transferred into the growth chamber and maintained under 12 h light of 600 - 1200 lx. For shoot growth and rooting in turmeric, Keshavachandran and Khader (1989) used culture conditions with pH 5.8, and temperature of 26 ± 1°C, with 12 h light (1000 lx). On the other hand, Wannakrairoj (1997) had successfully

cultured his patumma explants under 16.9 - 22.1 $\mu mol.m^{-2}s^{-1}$ of daylight fluorescent lamp at temperatures of 28 ± 2°C or 26 ± 1°C.

Polyploidy in Aromatic and Medicinal Plants

Among the wide range of benefits gained from polyploid induction, increasing sizes of different plant parts, upgrading heterosis and vigor, and improving resistance and/or tolerance to different biotic and abiotic stresses could be cited as the prime ones. However, the specific effects show significant differences with the species in question, the degree of heterozygosity incorporated, the ploidy level induced, etc. (Sanford, 1983).

The positive effects of polyploidization were reported for improvement of different aromatic and medicinal plant species. The technique is used for creating genetic variability that could bring about invaluable changes in the chemical composition of plants under this category, as this is mostly the prime target of improvement in these crop species. It can also change the percentage of essential oils, alkaloids, hydrocarbons, and other bioactive substances. According to Raev *et al.* (1996), the efficiency of induced polyploidy in increasing essential oil yields could be associated with increased size of flowers, inflorescence, and leaves, as well as other oil producing organs.

In colchicine-induced chamomile (*Matrica chamomilla*), tetraploids were more branched than the initial diploids, yielding 6% more heads and 9% and 7% more essential oil and chamazulene content of oil, respectively, and 38% heavier capitula (Glazova, 1970). In addition, the tetraploids exhibited thicker stems and increased resistance to lodging. Tsvetkov (1973) also reported success in production of *Salvia sclarea* tetraploids (2n = 44) using colchicine, which exceeded the initial varieties in their essential oil content. Comparisons were also made between tetraploid and diploid lavender lines and the former revealed larger number of oil glands, 16 – 66% more essential oil and 9% more esters than the latter (Rabotyagov, 1975). In their *in*

vitro synthesis of essential oils from *Mentha piperita*, Bricout *et al.* (1978) observed a 3-fold increase in essential oil from the use of colchicine at the level of 5×10^{-6} or 10^{-5}, which is predominantly ascribed to the formation of increased number of secretary glands.

Dijkstra and Speckmann (1980) also reported an increase in the oil and caravone content of autotetraploid caraway (*Carum carvi* L.) seeds by 35.6% and 6.9%, respectively, compared to the diploids. In other instance, Kapelev and Rabotyagov (1981) observed improvement in the essential oil yield of marigold after induction of polyploidy using a 0.1% colchicine solution for 12 h, hence obtained a considerable number of potential lines that exceeded the original lines in yield and essential oil content. As reported by Mekhraz *et al.* (1988), treatment of *Salvia sclarea* seeds with 0.1% colchicine for 24 h, resulted in mutant lines that revealed rapid growth and flowering, and in some cases an increased fresh matter yield and essential oil content as compared to the control. Similarly, during their study on polyploid induction in *Salvia miltiorrhiza*, Gao *et al.* (1996) observed an increase in the major chemical compound, tanshinones, in the autotetraploids as compared to the control.

According to Ramachandran and Nair (1992), tetraploid ginger (*Zingiber officinale*), obtained from treatment of sprouting buds on the rhizome with a 0.25% colchicine solution for several hours, gave rise to more vigorous plants with larger rhizomes and higher yields than the diploid parent. Likewise, Nakasone *et al.* (1999) observed gingerol content, antioxidant activity of their methanol extracts and pungency intensity of tetraploid ginger to be higher than that in the diploid relatives. Pank *et al.* (1999) also reported vigorous growth of polyploid peppermint lines, with accelerated generative development, increased essential oil content and considerable changes in essential oils composition than those with lower chromosome number. In another instance, studies made on diploid and tetraploid chamomile showed considerable variation in their chemical composition, such that some components were increased with polyploidy while others reduced (Pekic *et al.*, 1999). Adaniya

and Shirai (2001) also reported their success in obtaining significantly higher pollen fertility and germinability in the tetraploid ginger clones than the diploids.

Polyploid induction

In plants, chromosome doubling may occur spontaneously or may be induced by treatment with antimitotic agents, although the former is very rare in nature. According to Predieri (2001), unlike those of the physical ones, chemical mutagens could result in relatively more gene mutations rather than causing chromosomal changes. In the course of polyplodization, the chemical mutagens are mainly involved in inhibiting the formation of spindle fibers and arresting mitosis at the anaphase stage (i.e. the stage at which the chromosomes were multiplied but cell division had not yet taken place) resulting in polyploid cells.

To achieve improved conversion rates, it is essential to develop an efficient system of induction for the plant species under question, i.e. selection of effective antimitotic agents, identifying the most responding plant parts for treatment and devising efficient way of treating the plants (van Duren *et al.*, 1996, Kermani *et al.*, 2003).

1. Chemical agents

Of the different types of chemicals that are currently used to accomplish this task of arresting mitosis and chromosome doubling, colchicine is the primarily identified and by far the most commonly and widely used chemical mutagen (Sanford, 1983; Thao *et al.*, 2003). Colchicine has been used at concentrations of 0 - 1% (2.5mM) to obtain tetraploid banana clones using shoot tips immersed in a liquid MS medium added with the chemical. Colchicine treatment of banana at the rate of 0.5% (1.25mM) added with 2% dimethylsulfoxide (DMSO) for 2 h gave the best results, in which 30% of the treated shoot tips were solid tetraploids (Predieri, 2001). Ross *et al.* (2000) also reported the colchicine technique to be extremely effective

resulting in 19 tetraploid and 5 mixoploid, of the 29 lines tested in *Buddleia globosa*. However, colchicine treatment of *in vitro* cultured *Miscanthus sinensis* plantlets only resulted in few tetraploid shoots and a relatively high percentage of ploidy chimeras (Petersen *et al.*, 2003).

Although colchicine has been an important reagent for chromosome doubling in association with plant breeding programs, it has several drawbacks in that it often results in low conversion rates, and unwanted mutant and chimeric plants, which are unsuitable for vegetative multiplication (Chauvin *et al.,* 2003). Besides, as reviewed by Thao *et al.* (2003), several side effects such as sterility, abnormal growth and morphology, chromosome losses or rearrangements and gene mutation had also been reported from the use of colchicine in different crop species. However, most people still prefer to use colchicine for polyploid induction, be it under *in vivo* or *in vitro* conditions.

Colchicine is also stated highly toxic to animals, as it binds specifically to animal tubulin dimmers, in contrast to its much lower affinity to plant tubulins (Morejohn *et al.*, 1987, Wan *et al.*, 1991; Petersen *et al.*, 2003). Consequently, concentrations at mM level are usually needed to inhibit plant cell division and induce chromosome doubling unlike the μM concentrations that are capable of affecting microtubule-associated processes in animals' mitosis and meiosis (Hart and Sabnis, 1976). Therefore, it is usually recommended to reduce the use of colchicine, especially the dry compound, as much as possible or its substitution with other antimitotic agents, with less toxicity (Petersen *et al.*, 2003).

A number of other mitotic inhibitors, including oryzalin, trifluralin, amiprophos-methyl, and N_2O gas had been forwarded and used for chromosome doubling, but with varying results (Taylor *et al.,* 1976; van Tuyl *et al.,* 1992; Bouvier *et al.*, 1994). Lignowski and Scott (1972) reported successful inhibition of mitosis in onion and wheat root tips after the application of Trifluralin, which affected mitosis in the same manner as that of colchicine. Upadhyaya and Nooden (1976) also observed a

similar pattern of inhibition of elongation, swelling in the elongation zone, depolarized cell enlargement, disruption of cell differentiation and polyploidization in maize seedling roots, by applying colchicine and various dinitroaniline herbicides. Thus, suggested that colchicine and the dinitroaniline herbicides had a similar action at the molecular level. Likewise, Beaumont and Widholm (1993) succeeded in using the antimicrotubular herbicide pronamide for increasing the chromosome numbers of haploid maize callus.

As reported by Chauvin *et al.* (2003), oryzalin (3,5-dinitro-N^4N^4 dipropylsulphanilamide) is a persistent herbicide (developed from the toluidine chemical family from Dow AgroSciences, USA) in agricultural practices. It acts as a germination inhibitor of weed seeds and is effective against graminaceous and some dicotyledonous plants. Therefore, it has been successfully used for pre-emergence weed control in different crops, including cotton, soybean, wheat and oilseeds (Anthony and Hussey, 1999). Oryzalin shows a strong binding affinity to plant tubulins and has a high microtubule depolymerising effect, which is similar with that of colchicine (Morejohn *et al.*, 1987) but at lower concentrations. Therefore, it has been tested on a number of economically important agricultural and horticultural crops and proved more efficient in doubling chromosomes (van Tuyl *et al.,* 1992; Tosca *et al.,* 1995; van Duren *et al.*, 1996; Chauvin *et al.*, 2003; Thao *et al.*, 2003).

Oryzalin was very effective in inducing chromosome doubling in maize callus, although it severely inhibited the growth of regenerable callus and plant regeneration (Wan *et al.*, 1991). Likewise, van Tuyl *et al.* (1990) reported their success on *in vitro* chromosome doubling of Lilium with oryzalin treatment and stated this particular chemical to be less inhibitory to regeneration than colchicine. Besides, it was also more effective in doubling chromosomes than colchicine at lower concentrations (0.01 - 0.001% vs. 0.1%). Similarly, *in vitro* treatment of Lilium and Nerine with 0.001 - 0.01% oryzalin was indicated as less inhibitory to regeneration, giving rise to a higher number of polyploid plants than colchicine treatment, for which a 10-fold higher concentration was needed (van Tuyl *et al.,* 1992).

As reported by Bouvier *et al.* (1994), the efficiency of inducing chromosome doubling in haploid apple shoots under *in vitro* condition was studied using the two antimitotic agents, colchicine and oryzalin, each at three levels (i.e. 0.025, 0.25 and 1.25 mM for colchicine and 5, 15 and 30 μM for oryzalin). Therefore, it was observed that oryzalin could be a better choice than colchicine for chromosome doubling on haploid apple shoots *in vitro*. Tosca *et al.* (1995) also reported oryzalin to be as efficient as colchicine for chromosome doubling in Gerbera (*Gerbera jamesonii*), and even superior to the latter mainly because of its lower phytotoxicity and the absence of long-term effects. Besides, longer treatment of oryzalin was also indicated to be effective in the diploidization of a larger number of cells, while reducing the problem of chimera.

According to van Duren *et al.* (1996), culturing shoot tips of diploid banana clones in liquid medium supplemented both with 5.0 mM colchicine for 48 hours or 30 μM oryzalin for seven days, both in combination with 2% (v/v) DMSO, gave a high frequency (23.1% and 29.1%, respectively) of solid tetraploids in the fourth vegetative generation. Besides, they stated oryzalin at lower concentrations to be less toxic and highly effective, producing higher numbers of solid tetraploids than that of colchicine. Similarly, comparing the chromosome-doubling and embryo-forming effectiveness of colchicine with that of the microtubule depolymerizing herbicides trifluralin, oryzalin and amiprophosmethyl (APM) in *Brassica napus*, Hansen and Anderson (1996) observed similar effects from the use of these three herbicides, but at far lower concentrations (100 times lower than colchicine).

In Rhododendron hybrids, oryzalin was more efficient than colchicine in inducing polyploidy. The biggest proportion of solid tetraploids (18.2% of the survived plants) was obtained from the 24 h treatment with 0.005% oryzalin (Väinölä, 2000, Väinölä and Repo, 2001). In ornamental *Alocasia,* oryzalin applied at 0.01% for 24 h gave the best result with four tetraploids out of 26 explants examined (15.4%) (Thao *et al.*, 2003).

On the other hand, Chauvin *et al.* (2003) reported the effectiveness of oryzalin in chromosome doubling of potato cells, although 50 - 100% of the tetraploid regenerants turned to be chimeric when plants were acclimatized after *in vitro* stage. On the other hand, Petersen *et al.* (2003) reported oryzalin treatment (30, 60, or 120 μM) of single *in vitro* shoots of *Miscanthus sinensis* for 18 h to be severely toxic, resulting in survival of a few shoots from the lowest concentration. In general, survival of the explants after colchicine or oryzalin treatments mainly depends on the chemical concentration and duration of the treatment. Thus, in almost all cases, higher chemical concentrations and longer treatment durations reduced survival of treated explants (Kadota and Niimi, 2002; Thao *et al.*, 2003).

On top of these, several studies revealed the effective role of DMSO as a carrier agent during treatment of plants with chemical mutagens (van Duren *et al.*, 1996; Predieri, 2001; Petersen *et al.*, 2003; Thao *et al.*, 2003). Therefore, the use of DMSO was mostly recommended (Hamill *et al.,* 1992; van Duren *et al.*, 1996) to gain effective results from the use of chemical mutagens. Thus, DMSO is commonly used with the chemical antimitotic agents to improve their efficacy.

2. Methodology of polyploid induction

The other important aspect to consider for succeeding in the endeavors of polyplodization is selection of the most effective chromosome doubling method that could give superior results (Petersen *et al.,* 2003). According to Chakraborti *et al.* (1998), *in vivo* treatment of different plant parts (germinating seeds, seedlings, and vegetative buds) had been widely used in the past for polyploid induction. However, in all cases, the success of tetraploid induction was low (7 - 22%). Moreover, the treatments were lengthy, laborious and uneconomical, due to a greater consumption of antimitotic chemicals for effective treatment of the target plant parts. Besides, the technique was usually associated with the production of higher rates of mixoploids.

On the contrary, the *in vitro* technique has a potential for improving efficiency of mutation induction in several aspects, as it offers a wide choice of plant material for treatment (*in vitro* axillary buds, organs, tissues, and cells), which are more suitable, as compared to the *in vivo* treatment of buds (Predieri, 2001). Besides, the *in vitro* technique enables induction of tetraploids under controlled conditions, hence enables development of a suitable protocol for the maximum recovery of tetraploids, as well as economic use of the chemicals (Chakraborti *et al.*, 1998). As stated by Ross *et al.* (2000), success of *in vitro* methods over earlier *in vivo* methods doubtlessly could be associated to some extent on the thinner cuticle (which results from the constant high humidity conditions of tissue culture), and the scope of bathing the explants in the solution in the course of treatment. Moreover, the controlled culture conditions, temperature and photoperiod could also favor the synchronous division of meristematic cells and resulting in a significant reduction of mixoploids among the tetraploids induced under *in vitro* conditions, thereby contributing to the exceptionally high recovery of solid tetraploids (Chakraborti *et al.*, 1998).

According to Ahloowalia (1998), the *in vitro* technique also enables rapid execution of the propagation cycles of subculture aimed at separating mutated from non-mutated sectors (dissolving a chimera to obtain homo-histonts). However, despite its potentials and the wide array of researches carried out on the subject, the combination of mutation induction and *in vitro* techniques has not yet been fully exploited for different crop species (Predieri, 2001).

Similar to other crop species, both *in vivo* and *in vitro* treatments of plant materials are currently used for the induction of polyploidy in different aromatic and medicinal plants. However, from their polyploidization studies in onion, Polumordvinova *et al.* (1985) described the *in vivo* method as inefficient when compared to the *in vitro* one. Likewise, Schifino and Fernandes (1987) reported high frequency of chimeras in *Trifolium* to be associated with *in vivo* colchicine treatment, while Cohen and Yao (1996) stated the *in vitro* treatment with colchicine to be highly efficient for inducing polyploidy in *Zantedeschia*.

On top of these, some people stated the use of liquid media (Hamill *et al.*, 1992) as very important, while others (Adaniya and Shirai, 2001) recommended solid media to be much more effective for *in vitro* induction of polyploidy.

3. Explants for *in vitro* polyploid induction

Different workers treated different plant parts to succeed in their work of *in vitro* polyploid induction. Sanford (1983) has stated seed treatment to be effective only in clones that are known to breed true through seed and hence recommended antimitotic chemical treatment of axillary meristems in plant species that behave differently. Therefore, in most cases, it is common to treat callus, shoots, buds, etc. during *in vitro* polyploidization.

In line with this, Chen and Goeden (1979) obtained over 50% success by treating diploid callus (2n = 22) of daylily (*Hemerocallis flava)* with 0, 10, 20, or 40 mg l^{-1} colchicine in MS medium supplemented with 1 mg l^{-1} of 2,4-D and kinetin. It was the 20 mg l^{-1} colchicine that appeared to be the most effective. Somatic autotetraploidy was induced in rice by treating callus with various concentrations of colchicine and the maximum induction rate (45.5% and 56.3%) was obtained using colchicine concentrations of 300 and 500 mg l^{-1}, respectively. The mean induction rate was about 10 times than that of the controls (sprouting seeds) (Huang *et al.*, 1995). Calli from leaves and young stems of *Lycium chinense cv. Ningji-1* were also treated with 0.15, 0.2 or 0.25% colchicine and 2% DMSO before being transferred onto MS medium supplemented with 0.5 mg l^{-1} BA and 1 mg l^{-1} IAA. Regenerated plantlets after 25 and 30 days were sub-cultured and multiplied in order to establish polyploid clone lines. Thus, induction of autotetraploidy in Chinese wolfberry from calli was reported to be a simple, rapid and reliable approach, whereby satisfactory results were obtained even from the lowest colchicine concentration tested (Wang *et al.*, 1998). Similarly, results of a study conducted on *Scutellaria baicalensis* indicated the possibility of inducing autotetraploids through addition of colchicine to the culture medium, or by immersing callus in 0.2% colchicine solution prior to culture. The latter method proved effective inducing 40% autotetraploids (Chen *et al.*, 2000).

However, Huang (1983) used two techniques of colchicine application in his work on Lilium. In the first case, a piece of cotton soaked with 0.05% colchicine solution was placed in the center of *in vitro* cultured plantlets, followed by removal of leaf segments to be cultured on MS medium with 0.2 mg l^{-1} NAA and 2 mg l^{-1} BA. In the second instance, bulblet scales of *in vitro* cultured plantlets were placed in colchicine solution of 0.05% for 24 or 0.2% for 3 h.

Likewise, a positive response was obtained in clover (*Trifolium* spp.) hybrids from the *in vitro* method, whereby axillary meristems of the hybrids were cultivated on a shoot proliferation medium added with 0.1% colchicine for 48 or 72 h, with or without pre-culture (Anderson *et al.*, 1991). In other instance, Tonpayom (1994) succeeded in producing gerbera tetraploids (2n = 4N = 100) using MS medium supplemented with 0.4% colchicine for 5 days. Similarly, Cohen and Yao (1996) tried to induce chromosome doubling by growing rapidly multiplying shoots of *Zantedeschia* on culture medium containing 0.05% colchicine for 1, 2 or 4 days. Thus, reported the possibility of producing tetraploids with no or minimal genetic variation through such a treatment of multiplying shoots *in vitro*, compared to colchicine treatment of callus or suspension cells.

Hamill *et al.* (1992) treated their *in vitro* cultured banana shoot tips with colchicine at concentrations of 0, 0.05, 0.25, 0.5, 0.75 and 1.0% w/v (added directly into 125 ml Erlenmeyer flasks containing MS medium before autoclaving). Therefore, best results were obtained from those explants treated with a 0.5% (w/v) colchicine solution for 2 hrs under aseptic condition, whereby over 30% of the shoot tips treated revealed autotetraploidy. Van Duren *et al.* (1996) also used actively growing 3 - 5mm long shoot tip meristems of diploid banana for *in vitro* induction of polyploidy using colchicine and oryzalin. They added a 1 mM solution of oryzalin and 0.1 M solution of colchicine after filter sterilization to a liquid medium to reach a final concentration of 15, 30 and 60 μM for the former, and 2.5, 5.0 and 10.0 mM for the latter with or without DMSO. The treatment period was set to 48 hours for colchicine and 7 days for oryzalin. Thus, obtained 23.1 and 29.1% non-chimeric tetraploids from the

treatments involving 5.0 mM colchicine and 30 μM oryzalin, respectively both added with 2% DMSO.

Salon and Earle (1998) reported their success in inducing polyploidy on shoots of gamagrass (*Tripsacum dactyloides* L.) initiated from embryo-derived callus of diploid origin. The shoots were subjected to *in vitro* colchicine treatment with different concentrations (0.01 – 0.025%), whereby the colchicine solution was prepared using distilled deionized water and later on filter-sterilized. However, colchicine at 0.05% level was reported to be phytotoxic, with no shoots surviving the treatment. According to Pinheiro *et al.* (2000), polyploidy was effectively induced in *Brachiaria brizantha* (a forage grass) using colchicine treatment at 0.01% for 48 hours. Here, basal segments of *in vitro* regenerated plants were inoculated with colchicine, which was added into the autoclaved culture media after filter sterilization.

In other instances, buds were treated to attain this same objective of polyploidization. To this end, Li *et al.* (1999) compared the effectiveness of colchicine and the dinitroanilines ethalfluralin and oryzalin for their *in vitro* chromosome doubling of watermelon (*Citrullus lanatus*). Thus, single buds were isolated and treated with colchicine (100, 500, 1000 or 1500 μM) or dinitroaniline (5, 10, 50 or 100 μM) in liquid MS medium containing 3% sucrose and 10 μM BA. All cultures were incubated on a shaker (100 rpm) in the dark for 3, 6 or 9 days (also 30 days with colchicine). Treated buds were then transferred onto the same medium without colchicine or dinitroaniline, but containing 0.7% agar. All of the treated buds survived the chromosome doubling treatments, but some browning was observed on the edges of tiny leaves. In contrast to oryzalin, ethalfluralin at 10 μM induced 50% tetraploids in 9 days. With colchicine, 30 days was the most effective exposure time. After 30 days of exposure, the 1000 μM colchicine concentration resulted in the highest number of tetraploids, compared to the 100 μM concentration.

In line with plants of the aromatic and medicinal group, shoot tips of garlic (*Allium sativum*) were cultured on different media, including those with the addition of colchicine. The highest success in regeneration (92%) was obtained from the media without colchicine; however, only 64% of the shoot-tip explants formed plantlets after 3 months' culture including a week on medium containing colchicine. This proportion was maximized (86%) by the addition of DMSO to the medium containing colchicine. Of the 140 regenerated plants in the first clonal generation, 22.9% were tetraploid and 15% were diploid-tetraploid chimaeras. Both the diploid and tetraploid plants obtained from tissue culture were more vigorous and had larger bulbs than vegetatively propagated plants of the initial cultivar (Novak, 1983).

Tetraploidy was also induced by using apical and axillary meristems of the sterile interspecific hybrid *Mentha canadensis* X *Mentha aquatica* cultured in the presence of colchicine. These were later propagated by meristem culture. The polyploids differed from the initial material in different morphological characters, including that of their essential oil glands. The optimum period of incubation in the medium with colchicine was 1 - 4 days for axillary meristems and 7 - 10 days for apical meristems. The most effective colchicine concentration was 100 mg l^{-1}, which gave 16.7% polyploid plants (Bugaenko *et al.*, 1988).

Recently, Adaniya and Shirai (2001) studied the efficiency of *in vitro* induction of tetraploid ginger using shoot tip explants cultured on agar or in liquid MS medium containing 2.0 mg l^{-1} BA, 0.05 mg l^{-1} NAA and 0.2% (w/v) colchicine for 4, 8, 12, and 14 days. Therefore, treatment of shoot tip explants on agar medium containing 2.0 mg l^{-1} BA, 0.05 mg l^{-1} NAA and 0.2% (w/v) colchicine for 8 days was reported most effective for the production of tetraploid ginger.

4. Morphological changes due to polyploidy

Tetraploid Lilium plants were characterized with wider and thicker leaves, as well as deep green color and larger stomata (Huang, 1983). According to Sudharshan (1989), tetraploid cardamom plants exhibited a different morphological feature than their diploid counterparts. They were relatively robust, having thicker leaves, larger stomata and larger pollen grains than the diploids. With regard to their stomatal index, the number of stomata in the tetraploid leaves was lower than those of the diploid controls.

Several workers, (Speckmann *et al.,* 1965; Cohen and Yao, 1996; Chakraborti *et al.*, 1998; Kadota and Niimi, 2002) have stated morphological features related to stomatal character, i.e. stomatal density, length and index to be much more efficient predictors of ploidy levels. The stomatal size and density in micropropagated diploid and tetraploid plantlets of banana revealed significant differences. The tetraploids had considerably larger stomata than the diploids. Besides, the tetraploid plants had significantly less number of stomata per unit area than their diploid counterparts (Hamill *et al.,* 1992; van Duren *et al.*, 1996). However, Hamill *et al.* (1992) suggested the stomatal length as a very useful parameter for distinguishing tetraploids from diploids under *in vitro* condition. In lemongrass, reduced number of stomata and longer stomatal guard cells were suggested as indicatives of putative tetraploids (Meenattoor and Nair, 1993). Tetraploid mulberry plants had relatively large sized and less frequent stomata than their diploid relatives (Chakraborti *et al.,* 1998).

MATERIALS AND METHODS

Materials

Korarima seeds were collected from carefully selected mother clone (for yield and field performance) "Jimma local" at the Jimma Agricultural Research Center (JARC), Ethiopia, during the 2001/2002 cropping season. Fruits were dried under partial shade and brought to the Kasetsart University, Kamphangsaen campus, Thailand. Intact capsules were treated with 1 g kg^{-1} Benelate® 50 WP, kept in well-sealed polybags and stored in a refrigerator (4°C) till commencement of the study.

Rhizomes of korarima (Jimma local) obtained from the Jimma Agricultural Research Center (Ethiopia), and krawan brought from Chanthaburi Horticultural Research Institute (Thailand) were grown at a 50% shade house in the Kamphangsaen campus, Kasetsart University, Thailand, to initiate sprouting. Axillary buds (3 - 5 mm) were excised from actively growing rhizomes. The buds from the sprouting rhizome were thoroughly washed using laboratory detergent after removal of some outer bud scales. Subsequently, the buds were kept under running tap water for one and half hour. More scales were then removed, followed by washing with the detergent.

Methods

Five main experiments were conducted. The first experiment was undertaken on korarima, while the rest four were carried out on both korarima and krawan. The experiments were:

1. Effects of basal media strengths on *in vitro* germination of korarima seeds,
2. Effects of inorganic salt and coconut water on culture growth and development,
3. Effects of plant growth regulators on growth and development of plantlets,
4. Effects of casein hydrolysate, 2,4-D and BA on callus induction, and
5. Effects of colchicine and oryzalin on *in vitro* polyploid induction.

Experiment 1. *In vitro* germination of korarima seeds

Intact and healthy looking seed capsules were carefully selected from the stock and seeds were extracted, washed several times using laboratory detergent and were kept under running tap water for one and half hour. Then, they were thoroughly washed again with the detergent and put into pre-sterilized glass bottles with caps. All subsequent tasks were carried out under the laminar flow hood. Surface sterilization was undertaken using 70% ethanol for 3 minutes, followed by treatment with 20% and 10% Hyter® (ai: 6% sodium hypochlorite v/v) added with 2 ml l^{-1} Tween-80 for 10 and 5 minutes, respectively. To remove traces of chlorine, seeds were rinsed four times with sterilized distilled water.

After sterilization, seeds were transferred to the five germination media, including MS basal medium (Murashige and Skoog, 1962), modified MS media with macronutrients diluted to 1/2, 1/4, and 1/16 strength, as well as distilled water, as a control. The pH of the basal media was pre-adjusted to 5.7 prior to autoclaving. Twenty ml of each liquid germination media were dispensed into a 100 ml baby food jar embedded with a single layer of 5 X 6 cm cotton pad. The excess media was drained out from the jars. The bottles were then autoclaved for 20 minutes at 121°C (1.06 kg/cm^2). To avert the risk of losing cultures due to contamination, only one seed was cultured per bottle per replication. Ten replications were used in the experiment and the experiment was repeated three times.

Data collected

Seed germination, defined as radical protrusion (International Seed Testing Association, ISTA, 2003), was scored from 14 to 90 days after culture. Counts were made every other day till the 40^{th} day and at a five days interval, then after. Beside the germination percentage, both mean days to germination (MDG) (Nichols and Heydecker, 1968) and germination rate index (GRI) (Maguire, 1962; Steiner, 1990) were computed. The MDG and GRI were derived using the following formulae.

Mean Days to Germination (MDG) = $\frac{\Sigma (nt)}{\Sigma n}$

Germination Rate Index (GRI) = $\Sigma (n/t)$;

Where: n =number of newly germinated or emerged seeds at time t,

t = days from sowing.

Statistical analyses

To fulfill the basic assumptions of the analysis of variance (ANOVA) the percentage values obtained from the experiment were transformed using the arcsine transformation before analysis. Subsequently, all data were subjected to ANOVA and means were separated using the Duncan's Multiple Range Test (DMRT).

Culture initiation and establishment of axillary bud

For the next four main experiments, cultures were initiated from axillary buds of the nursery grown rhizomes as stated above. The buds were rinsed with 70% ethanol for 1 minute; followed by a two-step surface sterilization using 20 and 10% Hyter® (ai: 6% sodium hypochlorite v/v) added with 2 ml l^{-1} Tween-80 for 10 and 5 minutes, respectively. Then, explants were washed four times using sterilized distilled water and were further trimmed to remove dead and chlorine damaged tissues. Single explants consisting of a shoot tip with a small portion of the rhizome were cultured on a basal Murashige and Skoog (1962) (MS) medium for culture initiation, which later were used as source of explants for further experiments. Plantlets were subcultured every month on the same medium until the desired quantities were obtained for the subsequent experiments.

Unless specified otherwise, all media were added with 30 g l^{-1} sucrose and were gelled with 0.7% agar-agar after adjusting the pH to 5.7. All plant growth regulators were added to the medium prior to autoclaving and 20 ml of the respective medium was dispensed to each 100 ml baby food jar and covered with plastic cap.

The media were autoclaved for 20 minutes at 121°C (1.06 kg/cm^2). Cultures were incubated in the culture room at a temperature of 25 ± 2°C, under cool white fluorescent light of 28 μmol.m^{-2}.s^{-1} photosynthetic photon flux with 16 h photoperiod.

Each experimental unit consisted of two shoot tips (2 – 2.5 cm long) per bottle per replication. All experiments were conducted using ten replications in Completely Randomized Design (CRD) and all studies were repeated at least three times, though only data from the last two repetitions were used for analysis. Moreover, unless stated otherwise, all data were collected after eight weeks of culture. To fulfill the basic assumptions of ANOVA, some data were transformed using the appropriate technique, when deemed necessary. Analyses of the data from all experiments were conducted using SAS computer program Version 6.12 (SAS, 1995). All the four experiments were carried out independently for each plant species (korarima and krawan).

Experiment 2. Effects of inorganic salt and coconut water on culture growth and development

2.1. Effects of inorganic salt

Media with different concentration of inorganic salt, i.e. Murashige and Skoog (1962) (MS), a modified MS medium using half strength macronutrients (HMS), a modified MS medium using half-strength total nitrogen (HNMS), as well as Schenk and Hildebrandt (1972) (SH) medium were tested. All media were supplemented with 3 mg l^{-1} BA and 1 mg l^{-1} kinetin following the recommendation of Bajaj *et al.* (1993) for cardamom.

Data collected

- Number of green shoot apices (i.e. normal or axillary shoots and/or shoot buds produced),
- Number of leaves and primary roots, as well as average lengths (cm) of shoots and primary roots,
- Fresh and dry weight (g) of the plantlets.

Statistical analyses

All parameters were analyzed using the ANOVA, followed by the Duncan's Multiple Range Test (DMRT).

Rooting and acclimatization

An observation was also conducted on rooting and acclimatization of both species. Proliferated shoots were subcultured on a PGR-free MS medium for shoot elongation and rooting. Subsequently, 30 well rooted plantlets were washed under tap water to remove the adhering media and transferred to plastic pots of 10.1 cm diameter filled with a 1:1 mix of autoclaved river sand and peat moss for acclimatization. The plantlets were covered with plastic bags and kept under a 50% screen house for 7 days to maintain high level of relative humidity. Then after, the plastic bags were gradually opened to expose the plantlets for the external environment. The number and percentage of survived and acclimatized plantlets were counted and recorded after a month.

2.2. Effects of coconut water

Five levels of coconut water (CW), i.e. 0, 5, 10, 15 and 20% were examined. The basal medium used was a modified MS medium with 3 mg l^{-1} BA and 1 mg l^{-1} kinetin.

Data collected

Similar with that of experiment 2.1 above.

Statistical analyses

All parameters were analyzed using the general linear model (GLM) accompanied with contrast analysis, to come up with the best possible relationships between the different coconut water levels and the respective growth parameters.

Experiment 3. Effects of different plant growth regulators on shoot proliferation

This second experiment was conducted in two sub-experiments, including a preliminary one dealing with the effects of imazalil (IMA) in combination with thidiazuron (TDZ) or BA. The other study, however, involved full-fledged dose response experiments of TDZ in combination with BA, IMA or PBZ.

3.1. The combined effects of IMA with TDZ and IMA with BA

In this part, two simultaneous experiments were conducted using a 3 X 3 combination of IMA and TDZ or BA. Besides, a PGR-free medium together with a medium supplemented with 3 mg l^{-1} BA and 1 mg l^{-1} kinetin were also included as a blank and standard control, respectively. The basal medium used for these studies was a modified MS medium added with 5% CW.

3.1.1. Effects of IMA and TDZ

In this part, 0, 2 and 4 mg l^{-1} IMA were evaluated in combination with 0, 0.1 or 0.5 mg l^{-1} TDZ.

Data collected

Similar with that of experiment 2.1 above.

3.2.2. Effects of IMA and BA

In this case, IMA at 0, 2 and 4 mg l^{-1} were combined with BA at 0, 0.1 or 0.5 mg l^{-1} and compared.

Data collected

Similar with that of experiment 2.1 above.

Statistical analyses

All parameters were analyzed using ANOVA and the treatment combination that gave the highest shoot growth and development from the two simultaneous experiments were taken and compared with the blank and standard controls using the Student-Newman-Keuls' (SNK) mean separation test.

3.2. Synergistic effects of TDZ in combination with some other plant growth regulators

On top of the results from experiment 3.1, further experiments were carried out to evaluate the synergistic effects of TDZ in combination with benzyladenine (BA), imazalil (IMA) or paclobutrazol (PBZ). The basal medium used for all these studies was a modified MS medium with 5% CW.

3.2.1. Effects of TDZ and BA

The effects of TDZ at 0, 0.25, 0.5 and 0.75 mg l^{-1} in MS medium were evaluated in combination with 0, 1.5, 3 or 4.5 mg l^{-1} BA.

Data collected

- Number of green shoot apices (i.e. normal or axillary shoots and/or shoot buds produced),
- Number of leaves and primary roots, as well as average lengths (cm) of shoots and primary roots,
- Fresh and dry weight (mg) of the plantlets.

Statistical analyses

All parameters were analyzed using the GLM procedure accompanied with contrast analysis to establish an effective dose response relationship between the growth regulators and the specific growth parameter recorded. Means were also separated using DMRT.

3.2.2. Effects of TDZ and IMA

The effects of TDZ and IMA were further compared in a 4 X 4 factorial combination using TDZ at 0, 0.25, 0.5 and 0.75 mg l^{-1}, and IMA at 0, 2, 4 or 8 mg l^{-1} concentrations.

Data collected

Similar with that of experiment 3.2.1 above.

Statistical analyses

Similar with that of experiment 3.2.1. above.

Histological study

Histological sections were prepared and microscopic observations were made to determine if the tiny mass of shoots produced in the experiment resulted from embryogenesis or organogenesis. For the histological study, 0.5 - 1.0 cm sections of the callus were fixed in a 50% formaldehyde-acetic acid (FAA) solution and dehydrated through a graded ethanol and infiltrated with paraffin wax. Vertical sections (12 μm) were cut from the callus using a rotary microtome. The sections were stained with safranin and fast green, and then allowed to dry. Preliminary observations were made under a light microscope (with 200X magnification), and then pictures were taken using an automatic photomicrographic microscope at 1000X magnification.

3.2.3. Effects of TDZ and paclobutrazol (PBZ)

The combinations of 0, 0.25, 0.5 and 0.75 mg l^{-1} TDZ with 0, 1.5, 3 or 4.5 mg l^{-1} PBZ on a modified MS medium were also evaluated.

Data collected

Similar with that of experiment 3.2.1 above.

Statistical analyses

Similar with that of experiment 3.2.1. above.

Histological study

Similar with that of experiment 3.2.2. above.

Experiment 4. Effects of casein hydrolysate (CH), BA, and 2,4-D on callus induction

The combined effects of 1, 2 and 4 mg l^{-1} 2,4-D, and 0.1, 0.5 and 2.5 mg l^{-1} BA, with and without 1 g l^{-1} casein hydrolysate (CH) were evaluated together with a blank control. The basal medium used in this study was a modified MS medium with 18% CW and 2% sucrose following the recommendation of Srinivasa Rao *et al.* (1982).

Data collected

- % Callus induced
- Type of callus induced (i.e. compact, friable or amorphous)

Statistical analysis

Similar with that of experiment 1 above.

Experiment 5. *In vitro* polyploid induction

5.1. Polyploid induction

Five concentrations of colchicine (0, 125, 250, 375 and 500 μM) and oryzalin (10, 20, 40, 60 and 80 μM) in 2% DMSO were evaluated. Each treatment contained 10 replications and three explants were used per replication.

Stock solutions of both chemicals were prepared in DMSO, at concentrations of 0.25 M and 4000 μM for colchicine and oryzalin, respectively. In all treatments, including the control, 2% DMSO was used as an additive. The final concentration of DMSO in the media was adjusted to 2%. For all the ten treatments, PGR-free liquid

MS medium was prepared and autoclaved, in ten flasks, each containing an amount that can be adjusted to 400 ml after addition of the respective quantities of chemicals and DMSO supplement. After addition of the chemicals and DMSO supplement under aseptic condition, twenty ml of the liquid medium was dispensed into each flask. Three shoot explants from *in vitro* stocks were taken and cut to 10 – 15 mm and were put into each flask.

Flasks with explants in liquid medium were kept on a rotary shaker (100 rpm) and incubated under dark. After seven days, non-contaminated shoots were recorded and transferred to a PGR-free liquid MS medium and kept on the shaker for another two weeks. Then after, the surviving shoots were recorded and cultured on a shoot proliferation medium (i.e. 2 mg l^{-1} IMA and 0.5 mg l^{-1} TDZ) for eight weeks. Shoots were then transferred to MS medium supplemented with 3 mg l^{-1} BA and 1 mg l^{-1} kinetin for elongation and production of multiple shoots. Elongated shoots were then sub cultured three times at a monthly interval on a PGR-free MS medium for rooting and plantlet production.

5.2. Dete rmination of ploidy levels

At the end of the third cycle of subculture on a PGR-free medium, preliminary categorization of plantlets was carried out based on their morphology. These plants were further categorized using stomatal density, length and index following the procedures described by Hamill *et al.* (1992) and van Duren *et al.* (1996). However, final confirmation of their ploidy level was done through chromosome counts.

5.2.1. Morphological characters

Among the varied morphological characters that could be used to differentiate plants with different ploidy status, color and size of leaves, together with robustness of the stem were the ones that were given attention in the present experiment.

5.2.2. Stomatal characters

For the initial screening, a single fully developed leaf was sampled from each selected plant. The stomatal characters from the diploid control leaves were used as a base line to segregate the putative polyploid plants. To evaluate the stomatal characters of leaves, a small area (about 1 cm^2) on the lower epidermis of the leaf was smeared with a thin layer of transparent nail polish and left to dry for ten minutes. After that, the nail polish impression was swiftly removed using a piece of transparent adhesive tape. The tape with the epidermal layer impression was then placed on an object slide and observed under a light microscope at 400x magnification. The stomatal length was measured from each sampled field of view using an ocular-micrometer (400X magnification). Besides, the number of stomata and number of epidermal cells were counted and recorded within each field of view (1.0 mm^2) (Hamill *et al.*, 1992; van Duren *et al.*, 1996). In all cases, data were recorded from ten independent sampled spots of the nail polish impression. The stomatal index of the sampled leaf area was then calculated using the following the formula described by Weyers and Meidner (1990) and Willmer and Fricker (1996).

$$\text{Stomatal index} = \frac{\text{No. of stomata per unit leaf area (NSULA)} \times 100}{\text{NSULA} + \text{No. of epidermal cells per unit leaf area}}$$

Data analysis:

Simple statistics, including percentage, mean, standard deviation and coefficient of variation of the data were calculated and used to summarize the data.

5.2.3. Chromosome count

Root tip preparations and chromosome counts were made following the general procedures outlined by different workers (Singh, 1993; Fukui, 1996; Jahier *et al.*, 1996). Therefore, 0.5 – 1.0 cm of actively growing root tips were collected between 9:30 and 10:30 am, washed with tap water and pretreated in 0.002 M 8-

hydroxy quinoline and kept at 4°C in a refrigerator for 4 - 5 h. Pretreated roots were washed again and fixed in Carnoy's solution (i.e. 3 parts absolute ethanol mixed with 1 part glacial acetic acid) at 4°C for a day. Then after, roots were washed and transferred into 1 N HCl solution and macerated using hot water bath (60°C) for 5 min. Subsequently, the roots were kept in 70% ethanol at 4°C until observation.

For chromosome observation, roots were placed on glass slides and 1 - 2 mm of the root tips were cut and chopped into pieces. Roots were well squashed on the slide adding 1 - 2 drops of 1% aceto-orcine and then covered with a cover slip. The excess aceto-orcine solution was blotted with tissue paper and cells were further squashed gently with thumb. Slides were then put under a photomicroscope (Nikon Labphot-2, Japan) and pictures were taken using the 1000x magnification with a drop of oil. Therefore, chromosomes of the controls (diploids) and the putative polyploid plants were counted and compared. The chromosome count of each polyploid plantlet was repeated at least three times for confirmation.

RESULTS AND DISCUSSION

1. *In Vitro* Germination of Korarima Seeds

In vitro germination of korarima seeds was highly influenced by the composition of the basal medium. Basal medium with full strength MS salts resulted in the least germination percentage, longest mean days to germination (MDG) and the least germination rate index (GRI). Therefore, full strength MS medium was inferior for korarima seed germination, giving about 56% germination, as compared to the control (distilled water), which resulted in around 94% germination. Statistically no significant differences were observed between the control and all the different diluted concentrations of MS basal media tested (i.e. MS diluted to 1/2, 1/4 and 1/16 concentrations) (Table 1).

Similarly, seeds cultured on full strength MS medium took relatively longer time for germination (46.56 days) and recorded the least germination rate index (0.0816), both of which are indicators of least performance (Table 1). In general, the results from this study indicated media with low salt content to be the best for *in vitro* germination of korarima seeds. This finding could lay a foundation for future hybridization works in korarima involving embryo rescue techniques.

Hydrolysis of stored lipids in the endosperm is an important step in the germination process of seeds, which is mainly governed by the amount of water imbibed by the seeds. On the other hand, the rate of water imbibition during seed germination is entirely dependent on the osmotic potential of the internal and external environment of the seed. Therefore, culture media containing high concentrations of organic salts are characterized by high osmotic potential (George, 1993). This in turn results in reduced water content, and hence reduced metabolic activity within the seeds. Therefore, the reduced germination percentage and other related parameters recorded from the present study of korarima could be attributed to such increased osmotic potential levels within the MS medium.

These results were in agreement with that of Srinivasa Rao *et al.* (1982), who successfully germinated cardamom seeds on Went's medium, which is also a low salt medium. Pierik (1987) had also stated the suitability of a salt poor medium for orchid seed germination. However, the present result was in contrast to the findings of Almaz *et al.* (2000) who stated full strength MS medium to be the best for the *in vitro* germination of *Ensete ventricosum*, which is also a member of the order Zingiberales.

Table 1 *In vitro* germination of korarima seeds 90 days after sowing on five media formulations.

Medium [1/]	% G [2/]	MDG [3/]	GRI [4/]
MS	55.56b	46.56a	0.0816b
1/2-MS	85.00a	29.25b	0.1845a
1/4-MS	88.44a	27.83b	0.2160a
1/16-MS	95.31a	27.67b	0.2358a
DW	94.06a	28.09b	0.2256a
Prob.	0.0018	0.0319	0.0003
% CV	27.62	42.27	35.66

[1/] MS = Murashige and Skoog (1962), 1/2-MS, 1/4-MS and 1/16-MS =MS basal medium diluted to 1/2, 1/4 and 1/16 strength, DW= distilled water (control).

[2/] %G = percent germination; Transformed using the arcsine transformation before analysis.

[3/] MDG = mean days to germination

[4/] GRI = germination rate index.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

2. Comparison of Inorganic Salt and Coconut Water on Growth and Development

2.1. Effects of inorganic salt on growth and development

Formulations of basal media affected all shoot, leaf and root numbers, as well as shoot and root lengths of korarima. The highest number of shoots and leaves were obtained from the MS medium, while both HMS and HNMS media followed by the SH medium gave the highest root number. The shortest shoots were produced on the MS medium followed by the SH medium. However, korarima explants cultured on the MS medium produced significantly longer roots, while roots from the SH medium were the shortest (Table 2). These results of korarima shoot number are in accordance with the NO_3^- concentration of the different basal medium used ($y = 1.16x + 1.4$; $R^2 = 0.97$). The number of root and root length of korarima were inversely proportional to nitrate concentration ($y = -1.76x + 9.50$; $R^2 = 0.96$, and $y = -0.77x + 5.10$; $R^2 = 0.84$, respectively). On the other hand, both leaf number and shoot length of korarima seem to be related to the total nitrogen content of the respective medium used in the study ($y = 9.81x + 12.32$; $R^2 = 0.67$ and $y = 0.56x + 2.99$; $R^2 = 0.86$). The higher number of shoots obtained from the MS medium in this experiment could be indicative of the preference of korarima plants for nitrate-nitrogen.

Likewise, the composition of basal media significantly affected the numbers of shoot, leaf and roots, as well as root length of krawan. Non-significant effects were observed on the other parameters recorded, i.e. shoot length, as well as fresh and dry weights of *in vitro* plantlets. The highest number of krawan shoots were obtained from the MS medium followed by the SH medium. This could be ascribed to the relative concentrations of nitrate (NO_3^-) in the respective media ($y = 0.93x + 1.75$; $R^2 = 0.93$). The number of leaves, however, was observed to fairly relate to the total nitrogen content of the respective medium ($y = 1.2x + 7.1$; $R^2 = 0.52$). The root number and lengths showed fairly inverse relationship with the nitrate concentration in the basal medium ($y = -x + 8$; $R^2 = 0.61$ and $y = -1.45x + 10.52$; $R^2 = 0.41$,

respectively) (Table 3). Thus, relatively numerous and longer roots were obtained from both half MS macro and half MS nitrogen media.

Table 2 *In vitro* growth and development of korarima after eight weeks of culture on four media formulations.

Media [1/]	Shoot no.	Leaf no.	Root no. [2/]	Root lg. (cm) [2/]	Shoot lg. (cm)	FW (g)	DW (g)
MS	6.2a	13.5a	2.2b	1.92b	5.37a	0.843	0.094
HMS	2.8c	7.3b	7.3a	3.97a	4.45b	0.659	0.084
HNMS	3.4c	8.5b	6.6a	4.19a	4.34b	1.077	0.125
SH	4.8b	5.8b	4.3ab	2.66ab	3.45c	0.669	0.085
Prob.	0.0001	0.0001	0.0026	0.0077	0.0009	0.1294	0.1830
% CV	27.19	38.58	34.67	27.18	21.63	52.46	46.46

[1/] MS= Murashige and Skoog (1962), HMS= Half macro MS medium, HNMS= Half Nitrogen MS medium, SH= Schenk and Hildebrandt (1972).

Root lg. = Root length, Shoot lg. = Shoot length, FW=Fresh weight, DW =Dry weight.

[2/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

As nitrogen is one of the major constituents of many plant cell components, its deficiency inhibits plant growth. In addition to this total nitrogen content, the ratio of nitrate to ammonium (NH_4^+) is a very important aspect in nitrogen nutrition (Singh *et al.*, 1999; Ramage and Williams, 2002). Raab and Terry (1994) also reported the negative effects of ammonium nutrition on growth of *Beta vulagris*. Thus, as in most plant species, the relatively higher supply of nitrate-nitrogen within the MS medium exerted the profound effect on shoot growth of these plant species. In contrast to the reports of Bajaj *et al.* (1993) for cardamom, and Roy and Pal (1991) for *Costus specious*, the MS medium produced the highest number of korarima and krawan shoots than the SH medium in the present study. As optimal macronutrient levels of plants differ with genotype (Tornero and Burgos, 2000), the nutrient requirement

differences observed in korarima and krawan from the above two close relatives could be associated to their genetic makeup.

Table 3 *In vitro* growth and development of krawan after eight weeks of culture on four media formulations.

Media[1/]	Shoot no.	Leaf no.	Root no. [2/]	Root lg. (cm) [2/]	Shoot lg. (cm)	FW (g)	DW (g)
MS	5.7a	13.3a	4.3b	5.57b	6.02	0.911	0.116
HMS	3.2c	8.9b	7.3a	10.26a	6.10	1.850	0.177
HNMS	3.0c	8.9b	6.5a	8.17a	5.62	0.587	0.077
SH	4.4b	9.3b	3.9b	3.59b	4.08	0.426	0.047
Prob.	0.0001	0.0206	0.0003	0.0001	0.0726	0.2771	0.1038
% CV	30.63	34.93	16.82	19.48	34.28	184.54	115.14

[1/] MS= Murashige and Skoog (1962), HMS= Half macro MS medium, HNMS= Half Nitrogen MS medium, SH= Schenk and Hildebrandt (1972).

Root lg. = Root length, Shoot lg. = Shoot length, FW=Fresh weight, DW =Dry weight.

[2/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

The present finding on shoot proliferation was also in accordance with the reports of several workers who stated a better plant growth and development with higher proportions of nitrate- to ammonium-nitrogen in plant nutrition (Goyal *et al.*, 1982; Chaillou and Lamaze, 2001).

Nitrogen is involved in the synthesis of different principal component of plant cells including amino acids, proteins, nucleic acids and chlorophyll that are indispensable in plant growth and development (Morot-Gaudry *et al.*, 2001), thus its deficiency results in stunted growth. As stated by Pilbeam and Kirkby (1992), nitrate (NO_3^-) is the most preferred nitrogen source for plant growth. Besides, the total nitrogen content, the relative concentration of NO_3^- and ammonium (NH_4^+) was

reported to exert a strong influence on plant growth and development by influencing the medium pH, which in turn determines nutrient availability (Niedz, 1994; Raab and Terry, 1994; 1995; Fracaro and Echeverrigaray, 2001; Jang *et al.*, 2003).

On top of the relative ratio of these two forms of inorganic nitrogen, the considerable reduced total nitrogen content in the rest three media formulations (½-MS, ½-nitrogen MS and SH) resulted in the production of fewer leaves than that of the MS medium. On the other hand, the findings on root number and length from the current experiments were in accordance with the reports of Drew *et al.* (1973), who observed fewer but longer roots in nitrate-deficient solutions during their work in barley. Similarly, Touraine *et al.* (2001) also associated this inhibition of root growth to high levels of tissue nitrate.

From the preliminary observation, both korarima and krawan were easily elongated and rooted when transferred to a PGR-free MS medium. Rooted plantlets were successfully acclimatized to the *in vivo* conditions through transfer to a 1:1 potting mix of river sand and peat moss. After seven days of retention under a high relative humidity condition and subsequent gradual exposure to the ambient environment, the percentage recovery of korarima plantlets was around 93% and that of krawan was more than 90%. In substantiating these, different workers have also reported successful rooting of plantlets using a PGR-free MS medium (Nirmal Babu *et al.*, 1992; Devi, 1999; Salvi *et al.*, 2002; Prathanturug *et al.*, 2003). Ease in rooting and acclimatization was also reported in other members of the family Zingiberaceae (Nadgauda *et al.*, 1978; Nadgauda *et al.*, 1983; Sajina *et al.*, 1997).

2.2. Effects of coconut water on growth and development

In vitro growth and development of korarima plants was highly influenced by the concentration of coconut water (CW) supplemented in the culture medium. Addition of CW to the culture medium resulted in higher numbers of shoot, leaf and root, as well as longer shoots and roots. It also gave higher dry weights of plantlets. The contrast analysis showed quadratic relationships between the CW concentration and all these parameters, except for shoot length and root number, which were linear (Table 4).

Table 4 *In vitro* growth and development of korarima after eight weeks of culture on a modified MS medium with coconut water.

% CW	Shoot no.	Leaf no.	Root no. [1/]	Root lg. (cm) [1/]	Shoot lg. (cm)	DW (g)
0	2.9	6.7	6.4	2.72	3.08	0.074
5	6.5	14.5	2.3	1.88	4.45	0.136
10	6.0	18.0	1.2	0.68	4.32	0.153
15	5.2	13.8	2.2	2.29	4.02	0.119
20	3.0	9.2	1.8	1.75	4.39	0.092
Prob.	0.0001	0.0001	0.0013	0.0310	0.0028	0.0038
% CV	39.06	39.41	44.17	37.05	20.34	41.77
Linear	0.5538	0.3851	0.0031	0.2572	0.0109	0.6579
Quadratic	0.0001	0.0001	0.0052	0.0467	0.0311	0.0002
Cubic	0.1501	0.4305	0.1153	0.2212	0.0116	0.2805

[1/] Transformed using square root transformation before analysis.

Root lg. = Root length, Shoot lg. = Shoot length, DW =Dry weight.

Similarly, CW supplement significantly affected the *in vitro* growth and development of krawan, except shoot and root lengths. Basal medium supplemented with 5% CW was the best for krawan shoot growth, i.e. shoot number, as well as dry weight (Table 5). The contrast analysis showed quadratic relationships between the CW concentration and shoot and leaf numbers, as well as dry weights of krawan.

However, root number had a linear relationship with the CW concentration, while root and shoot lengths were not significantly affected by the use of CW (Table 5).

Table 5 *In vitro* growth and development of krawan after eight weeks of culture on a modified MS medium with coconut water.

% CW	Shoot no.	Leaf no.	Root no. [1/]	Root lg. (cm) [1/]	Shoot lg. (cm)	DW (g)
0%	2.9	5.2	5.6	1.76	4.14	0.048
5%	5.3	10.3	4.6	2.91	6.12	0.098
10%	4.2	11.8	3.2	3.28	4.72	0.084
15%	3.7	8.3	3.9	2.05	4.04	0.083
20%	3.4	7.3	2.4	2.60	5.64	0.082
Prob.	0.0013	0.0058	0.0153	0.2718	0.1424	0.0022
% CV	31.47	45.89	27.89	29.89	43.54	33.41
Linear	0.6871	0.5776	0.0014	0.7432	0.6733	0.0541
Quadratic	0.0027	0.0008	0.8166	0.1231	0.9904	0.0071
Cubic	0.0042	0.1299	0.1299	0.1904	0.0117	0.0209

[1/] Transformed using square root transformation before analysis.

Root lg. = Root length, Shoot lg. = Shoot length, DW =Dry weight.

Several workers had reported the positive effects of CW supplement (5 - 20%) for the *in vitro* growth and development of different plant species in the family Zingiberaceae, including *Elettaria cardamomum* (Nadgauda *et al.*, 1983; Bajaj *et al.*, 1993), large cardamom (Sajina *et al.*, 1997), ginger (Brain and Richard, 1993) and turmeric (Shirgurkar *et al.*, 2001), as well. The present result was in constant agreement with the reports of Nadgauda *et al.* (1983) and Bajaj *et al.* (1993) for cardamom, Sajina *et al.* (1997) for *Amomum subulatum*, as well as Shirgurkar *et al.* (2001) for turmeric, who stated 5% CW as the optimal level for shoot multiplication.

In substantiating this, CW was reported to exert a strong growth stimulation effect on some plant tissue cultures. It is reported to contain several ingredients that are beneficial for growth and development of plants, including amino acids, organic

acids, nucleic acids, purines, sugars, sugar alcohols, vitamins, growth substances and minerals (George, 1993). It had exerted a stimulating effect on growth of isolated embryos (van Overbeek *et al.*, 1941) and carrot explants (Steward and Caplin, 1952). Among others, the unique growth promoting effect of CW could mainly be associated with its content of natural cytokinins (George, 1993).

3. Effects of Different Plant Growth Regulators on *In Vitro* Growth and Development

3.1. The combined effects of IMA with TDZ and IMA with BA

3.1.1. Effects of IMA and TDZ

Highly significant effects of IMA and TDZ were observed on all the *in vitro* growth and development parameters of korarima evaluated in this study, except for fresh and dry weights. The interactions of these two plant growth regulators were also highly significant, except for the number of leaves (Table 6). Nonetheless, both IMA and TDZ had individually enhanced the leaf production potential of korarima shoots. In general, the best result, with respect to shoot production (16.6 shoots/ explant), was obtained from the combined use of 2 mg l^{-1} IMA and 0.5 mg l^{-1} TDZ.

Similarly, the use of IMA and TDZ exerted a significant effect on shoot, leaf, and root numbers, as well as root and shoot lengths of krawan. Among these, however, only the numbers of shoot and leaf, as well as length of shoots were the particular parameters affected by the combined use of IMA and TDZ. The highest numbers of shoot and leaf were gained from the medium supplemented with 2 mg l^{-1} IMA and 0.5 mg l^{-1} TDZ, while the PGR-free medium gave the highest number of root, as well as the longest shoot and root. IMA showed no independent effect on root number, while TDZ exerted highly significant effects on all these five parameters. Inclusion of TDZ to the culture medium reduced root number and gave rise to comparatively shorter roots and shoots, as well. No statistical differences were observed between the effects of 0.1 and 0.5 mg l^{-1} TDZ on number and average length

of krawan roots. However, the use of 0.5 mg l^{-1} TDZ resulted in the shortest shoots (Table 7).

Table 6 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media with IMA and TDZ.

PGR	Conc. (mg l^{-1})	Shoot no.	Leaf no.	Root no. [1/]	Root lg. (cm) [1/]	Shoot lg. (cm)
IMA	0	7.83	13.48b	4.17	2.61	4.42
	2	9.67	17.07a	3.74	1.73	3.16
	4	10.98	16.04a	2.77	1.70	3.36
TDZ	0	2.25	8.34b	10.45	5.96	6.67
	0.1	10.28	18.12a	0.24	0.12	2.60
	0.5	15.52	19.76a	0.25	0.12	1.82
IMA X TDZ	0 X 0	2.21	6.32	12.21	7.58	7.58
	0 X 0.1	7.25	16.15	0.35	0.30	3.80
	0 X 0.5	13.75	17.60	0.35	0.19	2.04
	2 X 0	2.38	10.28	11.5	5.44	5.56
	2 X 0.1	9.26	17.42	0.10	0.00	2.37
	2 X 0.5	16.6	22.85	0.20	0.04	1.76
	4 X 0	2.16	8.53	7.68	4.83	6.82
	4 X 0.1	14.72	21.06	0.28	0.05	1.52
	4 X 0.5	16.26	18.79	0.21	0.12	1.65
Prob.	**IMA**	0.0001	0.0103	0.0001	0.0001	0.0001
	TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
	IMA X TDZ	0.0001	0.1415	0.0001	0.0001	0.0001
MODEL	**Prob.**	0.0001	0.0001	0.0001	0.0001	0.0001
	% CV	36.59	41.82	22.20	18.10	28.74

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using and the Student-Newman-Keuls' (SNK) mean separation test at 5% level of probability.

Root lg. = Root length, Shoot lg. = Shoot length.

Table 7 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media with IMA and TDZ.

PGR.	Conc. ($mg\ l^{-1}$)	Shoot no.	Leaf no.	Root no. [1/]	Root lg. (cm) [1/]	Shoot lg. (cm)
IMA	0	4.38	10.94	3.54	5.78a	6.72
	2	6.91	15.16	3.21	3.97b	5.23
	4	6.67	14.02	3.22	4.03b	5.57
TDZ	0	3.39	9.28	5.26a	6.81a	7.82
	0.1	6.64	14.17	2.95b	4.03b	5.40
	0.5	7.91	16.65	1.80b	2.97b	4.32
IMA X TDZ	0 X 0	2.83	9.06	6.44	9.42	9.42
	0 X 0.1	5.32	10.42	2.47	4.24	5.54
	0 X 0.5	4.94	13.39	1.78	3.78	5.26
	2 X 0	4.05	10.26	4.53	4.74	7.02
	2 X 0.1	7.10	15.74	3.21	4.19	5.49
	2 X 0.5	9.72	19.72	1.83	2.92	3.07
	4 X 0	3.24	8.41	4.82	6.38	7.02
	4 X 0.1	7.45	16.25	3.15	3.67	5.18
	4 X 0.5	9.06	16.83	1.78	2.20	4.63
Prob.	**IMA**	0.0001	0.0001	0.7811	0.0145	0.0001
	TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
	IMA X TDZ	0.0035	0.0202	0.3930	0.1306	0.0128
MODEL	**Prob.**	0.0001	0.0001	0.0001	0.0001	0.0001
	% CV	33.44	34.71	42.88	36.65	32.69

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using and the Student-Newman-Keuls' (SNK) mean separation test at 5% level of probability.

Root lg. = Root length, Shoot lg. = Shoot length.

3.1.2. Effects of IMA and BA

Except for dry weight of plantlets, all the parameters evaluated in this experiment of korarima were affected by the use of IMA and BA in the culture medium. The combined uses of both chemicals resulted in a synergistic effect on shoot number, as well as root and shoot lengths. However, the independent use of each chemical in the medium gave rise to higher number of leaves. On the other hand, the number of korarima roots was decreased with increasing concentrations of IMA. The addition of BA to the culture medium, however, increased fresh weight of plantlets. The highest number of korarima shoots (7.32 shoots/ explant) was obtained from the use of 4 mg l^{-1} IMA in combination with 0.1 mg l^{-1} BA (Table 8).

In a similar experiment involving different concentrations of IMA and BA in krawan, shoot number, as well as shoot and root lengths revealed statistical differences. However, interaction between the two chemicals was observed only on shoot number. The highest number of shoot was yielded from the use of 2.0 mg l^{-1} IMA and 0.5 mg l^{-1} BA. Addition of IMA to the culture medium gave rise to shorter shoots and roots in krawan. On the other hand, inclusion of BA to the medium increased shoot number but reduced their length (Table 9). This was in agreement with the reports of Werbrouck and Debergh (1996).

Table 8 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media with IMA and BA.

PGR	Conc. (mg l^{-1})	Shoot no.	Leaf no.	Root no. [1/]	Root lg. (cm) [1/]	Shoot lg. (cm)	FW (g)
IMA	0	2.60	10.14b	10.58a	5.98	6.54	0.879
	2	3.57	13.34a	6.82b	4.92	5.68	0.925
	4	4.81	13.16a	5.70b	5.10	6.05	1.001
BA	0	2.57	9.98b	8.52	6.63	6.79	0.800b
	0.1	4.65	13.74a	7.58	4.88	6.11	1.011a
	0.5	3.76	12.88a	7.05	4.53	5.42	0.992a
IMA X BA	0 X 0	2.16	7.42	11.47	7.80	7.47	0.763
	0 X 0.1	2.67	10.56	10.11	5.37	6.78	0.821
	0 X 0.5	2.95	12.35	10.15	4.78	5.44	1.041
	2 X 0	2.58	11.05	8.00	6.21	6.45	0.822
	2 X 0.1	3.83	15.11	7.22	4.72	5.83	1.072
	2 X 0.5	4.32	13.95	5.26	3.82	4.78	0.889
	4 X 0	3.00	11.56	5.94	5.83	6.44	0.818
	4 X 0.1	7.32	15.47	5.53	4.56	5.75	1.133
	4 X 0.5	4.05	12.40	5.65	4.96	6.00	1.040
Prob.	**IMA**	0.0001	0.0009	0.0001	0.0005	0.0008	0.2779
	BA	0.0001	0.0004	0.2102	0.0001	0.0001	0.0123
	IMA X BA	0.0001	0.2670	0.4879	0.0206	0.0155	0.2287
MODEL	**Prob.**	0.0001	0.0001	0.0001	0.0001	0.0001	0.0319
	% CV	42.20	41.50	25.78	13.09	19.73	43.33

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using SNK at 5% level of probability.

Root lg. = Root length, Shoot lg. = Shoot length, FW=Fresh weight.

Table 9 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media with IMA and BA.

PGR	Conc. (mg l^{-1})	Shoot no.	Root lg. (cm) [1/]	Shoot lg. (cm)
IMA	0	3.42	6.11a	7.26a
	2	4.89	3.44b	6.04b
	4	4.46	3.20b	5.97b
BA	0	3.75	4.28	7.08a
	0.1	4.25	4.13	6.35b
	0.5	4.73	4.34	5.87b
IMA X BA	0 X 0	2.94	6.10	8.26
	0 X 0.1	3.53	5.84	7.04
	0 X 0.5	3.74	6.39	6.58
	2 X 0	4.11	3.62	7.00
	2 X 0.1	4.56	3.48	6.06
	2 X 0.5	6.00	3.22	5.07
	4 X 0	4.17	3.22	6.04
	4 X 0.1	4.68	3.05	5.94
	4 X 0.5	4.53	3.35	5.92
Prob.	**IMA**	0.0001	0.0001	0.0003
	BA	0.0001	0.9248	0.0029
	IMA X BA	0.0086	0.9885	0.2644
MODEL	**Prob.**	0.0001	0.0001	0.0001
	% CV	25.17	27.58	29.25

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using SNK at 5% level of probability.

Root lg. = Root length, Shoot lg. = Shoot length.

3.1.3. Combined analysis of growth and development using some plant growth regulators

The use of different types and concentrations of plant growth regulators exerted significant effects on the *in vitro* growth and development of korarima. In the present study, the combined use of 2 mg l^{-1} IMA and 0.5 mg l^{-1} TDZ (IMATDZ) gave the highest number of shoots and leaves. As compared to the PGR-free medium, culture medium added with this combination of phytohormones produced 7.5-fold shoots and 6-fold leaves (Table 10). On the other hand, the former gave in the highest number of roots (12.42/explant), as well as the longest shoots (7.93 cm) and roots (7.82 cm). Maximum fresh weight of korarima plantlet was recorded from both BA supplemented media [4 mg l^{-1} IMA with 0.1 mg l^{-1} BA (IMABA-1), and 3 mg l^{-1} BA with 1 mg l^{-1} kinetin (BAK)].

Table 10 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media with different plant growth regulators.

Medium [1/]	Shoot no.	Leaf no.	Root no. [2/]	Root lg. (cm) [2/]	Shoot lg. (cm)	FW (g)
PGRF	2.21d	6.56d	12.42a	7.82a	7.93a	0.716b
IMATDZ	16.6a	22.85a	0.20c	0.04c	1.76d	0.750b
IMABA-1	7.32b	15.47b	5.53b	4.56b	5.75b	1.133a
BAK	5.42c	11.05c	4.37b	4.51b	4.23c	1.066a
Prob.	0.0001	0.0001	0.0001	0.0001	0.0001	0.0043
% CV	34.46	41.85	29.49	11.38	20.77	40.35

[1/] PGRF = Plant growth regulator free medium; IMATDZ = imazalil (2 mg l^{-1}) and thidiazuron (0.5 mg l^{-1}); IMABA-1 = imazalil (4 mg l^{-1}) and BA (0.1 mg l^{-1}), BAK = BA (3 mg l^{-1}) and kinetin (1 mg l^{-1}).

[2/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using SNK at 5% level of probability.

Root lg. = Root length, Shoot lg. = Shoot length, FW=fresh weight.

The types of plant growth regulators used in this study of krawan had a highly significant effect on all the seven parameters evaluated. The highest number of shoots (9.72) and leaves (19.72) in krawan were obtained from the culture medium supplemented with 2 mg l^{-1} IMA and 0.5 mg l^{-1} TDZ. Besides, both root and shoot lengths also revealed significant differences based on the type of plant growth regulator supplement used in the culture medium. The longest roots (8.36 cm) and shoots (8.75 cm) were recorded from the medium devoid of plant growth regulators. In this experiment, higher fresh weight values were recorded from the media added with 3 mg l^{-1} BA and 1 mg l^{-1} kinetin (BAK), as well as with 2 mg l^{-1} IMA and 0.5 mg l^{-1} BA (IMABA-2). With regard to dry weight of plantlets, except for the BAK medium that gave the lowest value (0.061 g/explant), no significant differences were observed between the rest three treatments (Table 11).

Table 11 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media with different plant growth regulators.

Medium [1/]	Shoot no.	Leaf no.	Root no. [2/]	Root lg. (cm) [2/]	Shoot lg. (cm)	FW (gm)	DW (gm)
PGRF	2.74c	9.53b	5.00a	8.36a	8.75a	0.797b	0.104a
IMATDZ	9.72a	19.72a	1.83b	2.92b	3.07c	0.828b	0.094a
IMABA-2	6.00b	11.89b	4.44a	3.22b	5.07b	1.134ab	0.119a
BAK	5.53b	10.21b	3.00ab	2.90b	5.25b	1.346a	0.061b
Prob.	0.0001	0.0001	0.0005	0.0001	0.0001	0.0077	0.0059
% CV	27.56	35.52	33.42	25.27	24.61	53.25	52.98

[1/] PGRF = Plant growth regulator free medium; IMATDZ = imazalil (2 mg l^{-1}) and thidiazuron (0.5 mg l^{-1}); IMABA-2 = imazalil (2 mg l^{-1}) and BA (0.5 mg l^{-1}), BAK = BA (3 mg l^{-1}) and kinetin (1 mg l^{-1}).

[2/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using SNK at 5% level of probability.

Root lg. = Root length, Shoot lg. = Shoot length, FW=Fresh weight, DW =Dry weight.

Similar to these, Chand *et al.* (1999) observed more vigorous growth of taro explants cultured on medium supplemented with 2.6 μM (0.6 mg l^{-1}) TDZ than BA, even at the rates as high as and above 3.0 mg l^{-1}. Rai (2002) has also gained the highest rate of *Nothapodytes foetida* shoot multiplication on a modified MS medium containing 2.2 μM (0.5 mg l^{-1}) TDZ, as compared to those added with either BA or kinetin at similar or higher concentrations.

To date, there are substantial evidences (Fellman *et al.*, 1987; Mok *et al.*, 1987; Chen and Piluek, 1995; Malik and Saxena, 1992b; Murthy *et al.*, 1998) that confirm the efficacy of TDZ in the induction of axillary bud break and production of adventitious buds, at a relatively lower rate than the adenine type cytokinins. Huetteman and Preece (1993), and Murthy *et al.* (1998) stated TDZ as the most potent cytokinin of all the phenyl urea derivatives so far studied for plant tissue culture use. Thidiazuron was reported to induce organogenesis at much lower concentrations via a reduced dominance of the apical meristem. Different reports (Mok *et al.*, 1987; Murthy *et al.*, 1998; Ponchia and Zanin, 2000; Rai, 2002) had described the biological activity of TDZ to be higher than or comparable to that of the most active adenine-type cytokinins at unusually low concentrations.

These unique effects of TDZ were mainly ascribed to the induction of synthesis or accumulation of endogenous cytokinins (Capelle *et al.*, 1983; Thomas and Katterman, 1986). Moreover, the phenomenon was also stated to relate to a variety of factors including the increase in synthesis, decrease in catabolism (Laloue and Fox, 1989), or release of biologically active cytokinin molecules from non-active storage forms (Murthy *et al.*, 1998).

This peculiar response of cultured tissues was basically associated with the high cytokinin requirements of actively growing tissues. Such tissues need higher cytokinin supplements so as to compensate the degradation effects of cytokinin oxidase, the enzyme that is known to reduce the efficacy of natural cytokinins to a large extent. Though much less active, the adenine-type cytokinins such as kinetin and

BA are also known to be less susceptible to this enzyme than the naturally occurring cytokinins such as zeatin. However, TDZ and some other phenyl urea derivatives are resistant to oxidases, stable, and biologically active at lower concentrations than the adenine-type cytokinins (Mok *et al.*, 1987). It is this unique character of TDZ that makes it much more potent in its effect than most natural and synthetic cytokinins so far under use in plant tissue culture.

On the other hand, from their study on Araceae, Werbrouck and Debergh (1996) reported the strong influence of IMA on multiple shoot production effects of cytokinins, including TDZ. Inducing changes upon the metabolism of exogenously applied cytokinin was suggested as a possible mechanism to reason out the potentials of IMA in enhancing cytokinin actions. They also reported improvements in the efficacy of TDZ through its combined use with IMA, than other cytokinins, including BA, zeatin and mT (6-3(-hydroxybenzyl) adenine). From their subsequent work on *Spathiphyllum floribundum*, these same authors reported improved shoot induction from the use of BA through its combined use with IMA, but with diminished sizes (Werbrouck and Debergh, 1997). This report was in direct agreement with the current findings in korarima. In another report, Werbrouck and Debergh (1995) suggested these positive effects of IMA upon BA to be associated with its possible role in altering the metabolism of BA or its probable inhibitory effect on their catabolic enzymes.

In line with the length of roots and shoots, several authors (Murthy *et al.*, 1998; Chand *et al.,* 1999; Roussos and Pontikis, 2002) reported the suppression of shoot elongation due to the use of TDZ. According to Fratini and Ruiz (2002), production of high number of shoots by TDZ was mostly accompanied with reductions in root number, as well as length of shoots and roots. Therefore, the average length of regenerated shoots and rooting were stated to be inversely proportional to the number of shoots formed on TDZ and BA supplemented media, though with different degrees. According to Murthy *et al.* (1998) and Arinaitwe *et al.* (2000), this suppression of shoot length and root growth could be attributed to the

effects of TDZ in inducing accumulation of endogenous cytokinin. Moreover, others (Inoue *et al.*, 2001; D'Arth *et al.*, 2002) stated such reductions in root lengths to be indications of the plant's sensitiveness to higher levels of cytokinin.

However, the observed shoot stunting and root inhibition effects of TDZ were a non-permanent phenomena and could be overcome by transferring the explants to BA containing or PGR-free media (Chand *et al.*, 1999; Rai, 2002). Similarly, in the current experiments involving both species, the small sized shoots produced from TDZ supplemented media were easily elongated, rooted and successfully revived their vigorous growth after two or three subcultures on a PGR-free medium. The findings related to the reduced root number and length of korarima and krawan plantlets grown on IMA supplemented media were in agreement with that of Werbrouck and Debergh (1995; 1996).

3.2. Synergistic effects of TDZ in combination with some other plant growth regulators on shoot proliferation

3.2.1. Effects of TDZ in combination with BA

Similar to the results obtained from the aforementioned plant growth regulator experiments of korarima and krawan, shoot number and dry weight of korarima showed considerable increment with the use of TDZ in the culture medium. But in both parameters, values started to decline when the concentration was over 0.5 mg l^{-1}. Shoot length showed a consistent decrease across all levels of TDZ evaluated. Significantly higher shoot number and dry weights were obtained from the media having 3 mg l^{-1} BA. However, there were no statistical differences between using 1.5 or 3.0 mg l^{-1} BA on dry weight of plantlets. In this experiment, korarima shoot number and dry weight of plantlets exhibited quadratic relationships with the concentrations of BA and TDZ. However, the relationship between the BA and TDZ concentration with that of root number was negative linear. Shoot and root lengths of korarima also revealed negative linear relationships with the concentrations of BA and TDZ used in this experiment (Table 12).

Table 12 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media with TDZ and BA.

PGR	Shoot no.	Root no.	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
TDZ (mg l^{-1})					
0	2.77c	4.59a	4.27a	3.11a	82.95c
0.25	9.76b	0.51b	1.52b	0.47b	99.17b
0.5	11.31a	0.23c	1.19c	0.27c	117.59a
0.75	9.89b	0.15c	0.97d	0.16c	103.26b
BA (mg l^{-1})					
0	8.38c	2.51a	2.97a	2.14a	93.49b
1.5	8.96b	1.84b	1.89b	0.91b	111.67a
3.0	10.24a	0.74c	1.63c	0.62c	115.39a
4.5	6.18d	0.36d	1.43d	0.33d	82.62c
Prob.					
TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
BA	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ X BA	0.0001	0.0001	0.0001	0.0001	0.0001
% CV	16.26	32.03	22.77	31.80	20.59
Contrast					
TDZ Lin.	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ Quad.	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ Cubic	0.0001	0.0001	0.0001	0.0001	0.0001
BA Lin.	0.0001	0.0001	0.0001	0.0001	0.0009
BA Quad.	0.0001	0.3118	0.0001	0.0012	0.0001
BA Cubic	0.0019	0.0001	0.0001	0.0001	0.9023

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

The simultaneous use of TDZ and BA in the culture medium has considerably affected growth and development of korarima. The best medium for shoot proliferation and plantlet growth was the combination of 0.5 mg l^{-1} TDZ and 3 mg l^{-1} BA (13.89 shoots and 137.48 mg dry weight/ explant, respectively) (Fig. 1a). However, no significant differences in dry weight of plantlets were observed when 3.0 mg l^{-1} BA was combined with either 0.5 mg l^{-1} or 0.75 mg l^{-1} TDZ. Of all the levels evaluated, the highest number of roots (9.00/ explant), as well as the longest shoots (7.49 cm) and roots (7.61 cm) were obtained from the PGR-free medium (Fig. 1b). In general, the sole use of BA in the culture medium seems relatively better for shoot elongation than its combined use with TDZ (Table 13).

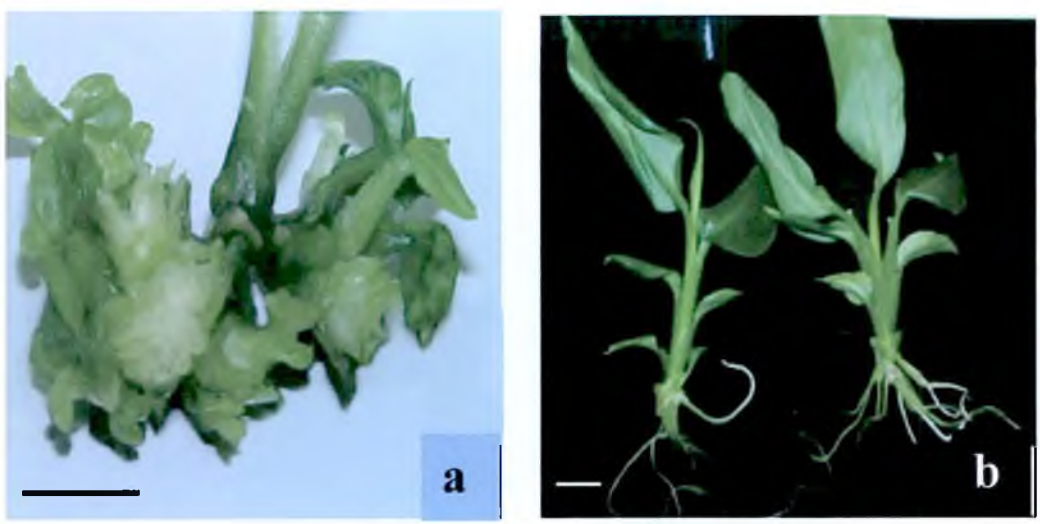

Figure 1 Korarima plantlets after eight weeks of culture on (a) modified MS medium with 0.5 mg l^{-1} TDZ and 3 mg l^{-1} BA (b) PGR-free medium (bar = 1cm).

Table 13 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media using TDZ in combination with BA (Mean ± SE).

TDZ (mg l^{-1})	BA (mg l^{-1})	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
0	0	1.89k ± 0.08	9.00a ± 0.40	7.49a ± 0.32	7.61a ± 0.23	86.66f ± 1.51
0	1.5	2.90j ± 0.16	6.40b ± 0.62	3.72b ± 0.14	2.57b ± 0.40	89.05ef ± 2.49
0	3.0	3.40j ± 0.11	2.30c ± 0.28	3.18c ± 0.09	1.74c ± 0.25	89.50ef ± 5.07
0	4.5	2.80j ± 0.14	1.10d ± 0.20	2.99c ± 0.05	0.98d ± 0.17	66.96g ± 3.00
0.25	0	9.65f ± 0.51	0.95de ± 0.20	2.16d ± 0.12	0.74de ± 0.14	96.17def ± 2.59
0.25	1.5	10.55def ± 0.39	0.55ef ± 0.15	1.47e ± 0.03	0.56def ± 0.15	107.53cd ± 2.59
0.25	3.0	11.44cd ± 0.28	0.28fg ± 0.14	1.29ef ± 0.05	0.38efgh ± 0.18	101.61def ± 6.21
0.25	4.5	7.33h ± 0.26	0.22fg ± 0.13	1.08fgh ± 0.08	0.14fgh ± 0.08	90.79ef ± 1.94
0.5	0	11.20cde ± 0.25	0.50fg ± 0.18	1.49e ± 0.02	0.54efg ± 0.19	102.71de ± 4.24
0.5	1.5	11.90bc ± 0.30	0.20fg ± 0.09	1.32ef ± 0.04	0.30fgh ± 0.14	130.96ab ± 4.54
0.5	3.0	13.89a ± 0.31	0.22fg ± 0.10	1.08fgh ± 0.04	0.25fgh ± 0.11	137.48a ± 5.44
0.5	4.5	8.50g ± 0.41	0.0g ± 0.0	0.86hi ± 0.07	0.0h ± 0.0	101.19def ± 6.21
0.75	0	10.15f ± 0.29	0.25fg ± 0.12	1.20efg ± 0.03	0.20fgh ± 0.09	87.74ef ± 6.47
0.75	1.5	10.50ef ± 0.29	0.20fg ± 0.09	1.04fghi ± 0.05	0.22fgh ± 0.10	119.14bc ± 3.47
0.75	3.0	12.70b ± 0.41	0.05g ± 0.05	0.89ghi ± 0.05	0.06gh ± 0.06	133.81a ± 7.25
0.75	4.5	6.20i ± 0.37	0.10fg ± 0.07	0.74i ± 0.09	0.18fgh ± 0.09	72.36g ± 6.15

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

Similarly, shoot and root number, together with shoot and root length, as well as well as plantlet dry weight of krawan were strongly affected by the use of TDZ and/ or BA in the culture medium. Krawan shoot number increased linearly with increasing levels of TDZ, though no significant differences were observed between the concentration of 0.5 and 0.75 mg l^{-1}. The highest plantlet dry weight was obtained from 0.5 mg l^{-1} TDZ. Moreover, the dry weight possessed a quadratic relationship with TDZ. On the other hand, the highest root number and length were recorded from the PGR-free medium. The use of 3.0 mg l^{-1} BA gave the highest number of shoots and dry weight of krawan plantlets. Both parameters exhibited a quadratic relationship with BA. However, root number as well as shoot and root lengths showed consistent reduction with increasing levels of each of the two bioregulators, revealing a negative linear relationship with these parameters (Table 14).

The combined use of TDZ and BA also revealed a synergistic effect upon all the parameters evaluated. The highest number of krawan shoots per explant (8.45) was obtained from the medium having 0.75 mg l^{-1} TDZ and 3 mg l^{-1} BA (Fig. 2a). No significant difference was observed in plantlet dry weight when 0.5 or 0.75 mg l^{-1} TDZ was combined with 3 mg l^{-1} BA. The highest number of roots (5.20 / explant), as well as the longest shoots (7.02 cm) and roots (6.02 cm) were obtained from the PGR-free medium (Table 15 and Fig. 2b).

Table 14 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media with TDZ and BA.

PGR	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
TDZ (mg l^{-1})					
0	3.28c	3.15a	4.96a	4.13a	76.51c
0.25	5.00b	1.99b	3.20b	2.66b	87.76b
0.5	5.81a	1.35c	2.62c	1.90c	94.55a
0.75	5.98a	0.62d	2.22d	1.30d	88.06b
BA (mg l^{-1})					
0	4.26c	2.97a	5.00a	3.86a	82.66c
1.5	5.14b	1.81b	3.17b	3.13b	90.16b
3.0	6.42a	1.40c	2.65c	1.77c	97.19a
4.5	4.22c	0.95d	2.22d	1.26d	76.74d
Prob.					
TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
BA	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ X BA	0.0001	0.0751	0.0001	0.0003	0.0001
% CV	27.87	26.56	22.88	26.50	15.64
Contrast					
TDZ Lin.	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ Quad.	0.0001	0.5811	0.0001	0.4877	0.0001
TDZ Cubic	0.0001	0.0001	0.0001	0.0001	0.0001
BA Lin.	0.0462	0.0001	0.0001	0.0001	0.0003
BA Quad.	0.0001	0.0058	0.0001	0.5150	0.0001
BA Cubic	0.0001	0.0001	0.0001	0.0001	0.0275

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

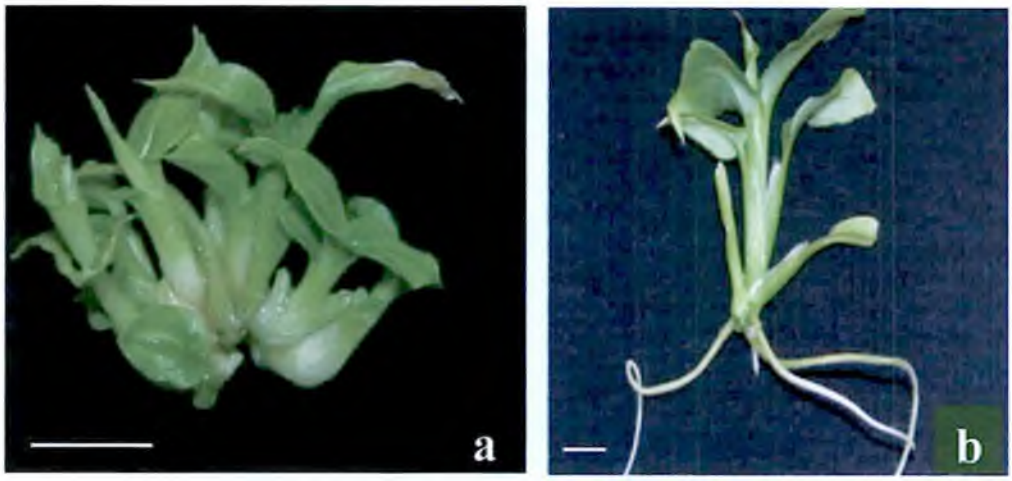

Figure 2 Krawan plantlets after eight weeks of culture on (a) modified MS medium with 0.75 mg l^{-1} TDZ and 3 mg l^{-1} BA (b) PGR-free medium (bar = 1cm).

In both experiments, inclusion of TDZ in the culture medium had effectively enhanced shoot proliferation of korarima and krawan. The improved shoot proliferation effects on the two species from the use of TDZ in the current study were in agreement with those of Salvi *et al.* (2000) and Prathanturarug *et al.* (2003) who reported a similar result in *Curcuma longa*. The significantly higher efficacy of TDZ observed in this study could be associated with its high potency in its cytokinin action. The biological activity of TDZ was stated higher than or comparable to that of most active adenine-type cytokinins at unusually low concentrations (Mok *et al.*, 1987; Murthy *et al.*, 1998; Rai, 2002). This could again be ascribed to its counteracting effect on the usual shortage of cytokinins in plant tissues, which results from their degradation by cytokinin oxidase, the enzyme that significantly reduces the efficacy of natural cytokinins (Mok *et al.*, 1987; Hare and van Staden, 1994a; 1994b). The adenine-type cytokinins, such as kinetin and BA, are also known to be less susceptible to this enzyme than their natural counterparts; though are much less active than the latter. However, compared to the adenine-type cytokinins, TDZ is much more resistant to oxidases, stable, and biologically active at lower concentrations (Mok *et al.*, 1987).

Table 15 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media using TDZ in combination with BA (Mean ± SE).

TDZ (mg l^{-1})	BA (mg l^{-1})	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
0	0	1.80j ± 0.16	5.20a ± 0.35	7.02a ± 0.16	6.02a ± 0.28	61.54h ± 3.03
0	1.5	3.40i ± 0.36	3.00bc ± 0.34	5.28b ± 0.30	5.15ab ± 0.53	80.65efg ± 2.91
0	3.0	4.15ghi ± 0.17	2.70cd ± 0.55	4.32c ± 0.29	3.30cd ± 0.40	89.20bcde ± 2.16
0	4.5	3.75hi ± 0.19	1.70def ± 0.22	3.24d ± 0.21	2.06efg ± 0.29	74.66g ± 4.55
0.25	0	4.61efgh ± 0.33	3.44b ± 0.32	5.62b ± 0.15	4.33b ± 0.21	88.22cde ± 2.92
0.25	1.5	4.90defg ± 0.27	1.95de ± 0.17	3.22d ± 0.23	3.31c ± 0.29	90.82bcd ± 2.05
0.25	3.0	5.65cd ± 0.34	1.40fg ± 0.28	2.09fg ± 0.10	1.51fg ± 0.31	94.10abcd ± 5.72
0.25	4.5	4.80defg ± 0.26	1.30efg ± 0.15	2.11fg ± 0.09	1.66efg ± 0.17	77.96fg ± 4.57
0.5	0	5.25def ± 0.30	1.90de ± 0.07	4.68c ± 0.09	2.91cd ± 0.23	96.26abc ± 2.50
0.5	1.5	6.55c ± 0.29	1.70def ± 0.23	2.15fg ± 0.11	1.77efg ± 0.26	98.62ab ± 1.29
0.5	3.0	7.45b ± 0.63	1.00fgh ± 0.10	1.92fg ± 0.05	1.59efg ± 0.17	103.16a ± 2.27
0.5	4.5	4.00ghi ± 0.30	0.80ghi ± 0.09	1.71g ± 0.07	1.31g ± 0.23	80.15efg ± 1.31
0.75	0	5.40de ± 0.34	1.40efg ± 0.18	2.75e ± 0.21	2.22def ± 0.31	85.16def ± 2.14
0.75	1.5	5.70cd ± 0.26	0.60hi ± 0.11	2.03fg ± 0.12	2.30de ± 0.31	90.56bcd ± 2.12
0.75	3.0	8.45a ± 0.31	0.50i ± 0.18	2.27f ± 0.10	0.67h ± 0.26	102.32a ± 1.77
0.75	4.5	4.35fghi ± 0.22	0.00j ± 0.00	1.83gf ± 0.08	0.0i ± 0.0	74.19g ± 3.39

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

In line with the current results, several studies (Murthy *et al.*, 1998; Chand *et al.*, 1999; Fratini and Ruiz, 2002) also confirmed suppression of shoot elongation due to the inclusion of TDZ in the culture medium. The negative effects of TDZ on shoot length were also further aggravated with the addition of BA to the culture medium (Werbrouck and Debergh, 1996). However, the independent use of BA in the culture medium has a relatively milder effect upon shoot length in the absence of TDZ (Babaoglu and Yorgancilar, 2000; Rai, 2002). These reports are also in accordance with the current findings.

The synergistic effects observed from the combined use of TDZ and BA in this study are in direct agreement with the reports of different workers (Sudarsono and Goldy, 1991; Khalafalla and Hattori, 1999) that described the benefits from the combined use of these two plant growth regulators. In substantiating the current findings, Nielsen *et al.* (1995) also reported the possible binding of BA and TDZ to a cytokinin-binding protein (CBP), which is a receptor having two different binding sites. Of these two, one site was stated to bind the adenine-type cytokinins, while the other is able to bind the phenylurea-types. Binding of an adenine-type cytokinin to CBP was reported to induce the well-known cytokinin effects, i.e. promotion of cell division and shoot formation, as well as regulation of various developmental events (Mok *et al.*, 2000). The change in cytokinin metabolism due to TDZ results in the induction of cytokinin autonomy by increasing the levels of endogenous cytokinins (Victor *et al.*, 1999a). Kende and Zeevaart (1997) also described TDZ as a non-competitive inhibitor of cytokinin oxidase, thereby increasing the efficacy of available cytokinins.

With regard to shoot elongation, several authors (Murthy *et al.*, 1998; Chand *et al.*, 1999; Arinaitwe *et al.*, 2000; Fratini and Ruiz, 2002; Roussos and Pontikis, 2002) had reported the suppression of shoot length due to the inclusion of TDZ in the culture medium. Tiwari *et al.* (2001) also obtained relatively large sized shoot buds from BA supplemented media, while the use of TDZ resulted in clusters of stunted

shoots. Besides, Nielsen *et al.* (1995) also reported enhanced shoot proliferation from the use of cytokinins to be accompanied with inhibition of their elongation.

This phenomenon could be attributed to the effects of TDZ in inducing endogenous cytokinin accumulation (Murthy *et al.*, 1998; Arinaitwe *et al.*, 2000). It could also be due to the comparatively higher cytokinin activity of TDZ even at a lower concentration (Arinaitwe *et al.*, 2000). In either way, the resulting higher cytokinin activity had a negative impact upon shoot elongation (Murthy *et al.*, 1998; Roussos and Pontikis, 2002). All these reports are in accordance with the findings from the current study of korarima and krawan.

However, these TDZ induced shoot stunting were successfully reverted in several instances with the transfer of explants to a PGR-free or BA containing medium (Chand *et al.*, 1999; Arinaitwe *et al.*, 2000; Rai, 2002). Similarly, the multiple shoots produced from the current experiments of korarima and krawan elongated, produced roots, and resumed normal growth on their subsequent subculture on a PGR-free modified MS medium. In both genera, the shoots produced on TDZ supplemented BA medium were of high quality (Figs. 1a and 2a) that easily elongated and produced roots upon transfer to a medium devoid of plant growth regulators.

3.2.2. Effects of TDZ in combination with IMA

In this experiment, TDZ has significantly improved the number of shoots and dry weight of korarima plantlets up to the third level (0.5 mg l^{-1}). The relationship between TDZ and these two parameters was quadratic. On the other hand, the use of TDZ in the culture medium has greatly reduced root number, root length, as well as shoot length in korarima. All these parameters showed negative linear relationships with the TDZ concentrations studied. Significant increments in shoot number and dry weight of korarima plantlets were also observed in cultures supplemented with 2 mg l^{-1} IMA. The relationships of IMA with korarima shoot number and dry weight were also quadratic. However, inclusion of IMA to the culture medium reduced root

number and shoot length, and hence resulted in a negative linear relationship with these parameters. However, IMA didn't exert any effect on the length of korarima roots (Table 16).

Table 16 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media with TDZ and IMA.

PGR	Shoot no.	Root no. [1/]	Root lg. (cm) [1/]	Shoot lg. (cm)	DW (mg)
TDZ (mg l^{-1})					
0	2.05d	9.22a	8.49a	8.19a	89.01c
0.25	13.31c	1.79b	0.94b	2.68b	98.40b
0.5	16.76a	0.49c	0.33c	1.58c	116.39a
0.75	14.73b	0.09d	0.04d	1.12d	94.42c
IMA (mg l^{-1})					
0	9.42c	3.21a	2.63a	4.08a	93.58bc
2	14.18a	3.01a	2.42b	3.51b	120.28a
4	11.77b	3.00a	2.44ab	3.07c	96.12b
8	11.18b	2.58b	2.50b	3.09c	88.16c
Prob.					
TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
IMA	0.0001	0.0067	0.2894	0.0001	0.0001
TDZ X IMA	0.0001	0.0001	0.0001	0.0755	0.0002
% CV	20.98	23.89	16.00	26.55	22.43
Contrast					
TDZ Lin.	0.0001	0.0001	0.0001	0.0001	0.9597
TDZ Quad.	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ Cubic	0.0001	0.0001	0.0001	0.0001	0.0001
IMA Lin.	0.0133	0.0119	0.0799	0.0651	0.0007
IMA Quad.	0.0001	0.0167	0.6651	0.1176	0.0001
IMA Cubic	0.0001	0.5842	0.4783	0.0001	0.0001

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

The combined use of these two chemicals showed a synergistic effect on the number of shoots, number of roots, root length and dry weight of korarima plantlets. The inclusion of these two bioregulators to the culture medium showed no significant effect on shoot length. The highest shoot multiplication rate for korarima (11.35-fold than the blank control) was obtained from the culture medium supplemented with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA, followed by the medium added with 0.75 mg l^{-1} TDZ and 2 mg l^{-1} IMA (18.78 shoots/explant). In this particular experiment, the inclusion of IMA didn't affect the number or length of korarima shoots on medium devoid of TDZ. On the other hand, the effects of IMA on both number of roots and root length were also minimal when TDZ was excluded from the culture medium (Table 17).

In krawan, the use of TDZ and IMA in the culture medium considerably affected all the growth and development parameters evaluated in the present study. Krawan shoot number showed a significant increase with the inclusion of TDZ in culture media. A similar trend was also observed for dry weight of plantlets, except at level four (0.75 mg l^{-1}) that gave the least value. Both shoot number and dry weight showed a quadratic relationship with TDZ. Shoot length on the contrary, showed a consistent decrease all along the concentrations of TDZ evaluated. Negative linear relationships were observed between TDZ and root number, as well as shoot and root lengths of krawan. The use of IMA in the culture medium also improved the number of shoots and dry weight of plantlets, while it reduced shoot length. The relationships between IMA with krawan shoot number and dry weight of plantlets were quadratic. However, the bioregulator had a negative linear relationship with krawan shoot length (Table 18).

Table 17 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media using TDZ in combination with IMA (Mean ± SE).

TDZ (mg l^{-1})	IMA (mg l^{-1})	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
0	0	1.70i ± 0.15	10.70a ± 0.47	8.41a ± 0.19	8.60ab ± 0.23	90.93efg ± 2.71
0	2	2.22i ± 0.10	8.72b ± 0.36	8.54a ± 0.30	9.03a ± 0.13	92.57efg ± 2.16
0	4	2.10i ± 0.12	9.00b ± 0.59	7.59b ± 0.26	7.83b ± 0.21	83.82fg ± 3.30
0	8	2.20i ± 0.17	8.40b ± 0.40	8.25a ± 0.30	8.56ab ± 0.29	89.09efg ± 3.99
0.25	0	11.50h ± 0.55	0.80ef ± 0.09	3.52c ± 0.02	0.70de ± 0.09	93.83defg ± 2.65
0.25	2	15.70de ± 0.66	2.70c ± 0.34	2.86d ± 0.34	0.74de ± 0.09	121.48b ± 5.91
0.25	4	14.60ef ± 0.57	2.20cd ± 0.35	2.36de ± 0.29	1.31c ± 0.20	93.67defg ± 4.78
0.25	8	11.22h ± 0.22	1.44de ± 0.28	1.90ef ± 0.24	1.03cd ± 0.14	83.06fg ± 2.91
0.5	0	13.22f ± 0.84	0.50fgh ± 0.14	2.27def ± 0.06	0.62ef ± 0.20	102.27cde ± 1.57
0.5	2	19.30a ± 1.11	0.80fg ± 0.27	1.72fg ± 0.19	0.29fgh ± 0.09	151.52a ± 13.87
0.5	4	16.70cd ± 0.47	0.50fgh ± 0.15	1.23gh ± 0.12	0.39fg ± 0.10	109.35bcd ± 7.20
0.5	8	17.56bc ± 0.84	0.11ih ± 0.08	1.14gh ± 0.12	0.0i ± 0.0	99.31cdef ± 2.58
0.75	0	11.89gh ± 0.48	0.28ghi ± 0.11	1.68fg ± 0.20	0.14ghi ± 0.05	87.57efg ± 1.92
0.75	2	18.78ab ± 0.56	0.11ih ± 0.08	1.19gh ± 0.14	0.05hi ± 0.03	111.94bc ± 3.91
0.75	4	13.89f ± 0.21	0.0i ± 0.0	0.89h ± 0.08	0.0i ± 0.0	97.80cdefg ± 1.06
0.75	8	14.40ef ± 0.54	0.0i ± 0.0	0.76h ± 0.02	0.0i ± 0.0	81.79g ± 1.74

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

Table 18 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media with TDZ and IMA.

PGR	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
TDZ (mg l^{-1})					
0	2.96c	5.98a	7.69a	6.92a	79.48b
0.25	5.88b	1.41b	2.63b	1.54b	74.34c
0.5	7.78a	1.15bc	2.05c	1.27b	87.62a
0.75	5.62b	0.82c	1.29d	0.56c	73.01c
IMA (mg l^{-1})					
0	4.19c	2.53a	3.84a	3.09a	90.50a
2	7.32a	2.11ab	3.33b	2.50b	91.70a
4	6.06b	2.27ab	3.14b	2.36bc	75.31b
8	4.71c	2.26b	3.13b	2.09c	56.10c
Prob.					
TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
IMA	0.0001	0.0765	0.0001	0.0001	0.0001
TDZ X IMA	0.0001	0.0768	0.0001	0.8976	0.0001
% CV	37.76	32.54	24.66	31.56	14.43
Contrast					
TDZ Lin.	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ Quad.	0.0001	0.0001	0.0001	0.0001	0.0007
TDZ Cubic	0.0001	0.0001	0.0001	0.0001	0.0001
IMA Lin.	0.7824	0.0269	0.0001	0.0001	0.0001
IMA Quad.	0.0001	0.2808	0.5372	0.4059	0.0001
IMA Cubic	0.0006	0.3342	0.9431	0.4295	0.0387

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability. Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

The combined use of these two bioregulators had a synergistic effect on the number and length of krawan shoots, as well as dry weight of plantlets. The highest shoot number (10.25 shoots/explant) and dry weight of plantlets (104.18 mg/explant) were obtained from the medium added with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA. When the medium was devoid of TDZ, no significant differences were observed in shoot number and shoot length between the different concentrations of IMA tested in this experiment (Table 19).

Similar to the present results, enhanced shoot multiplication from TDZ supplemented medium were observed in different herbaceous plant species including banana (Arinaitwe *et al.*, 2000), *Curcuma longa* (Salvi *et al.*, 2000; Prathanturarug *et al.*, 2003) and minor millet (Rashid, 2002). This could be attributed to the strong potency of TDZ, which could be related to its effect on the metabolism of endogenous cytokinins (Hutchinson and Saxena, 1996b).

On the other hand, Werbrouck and Debergh (1996; 1997) had observed enhancement of the multiple shoot producing effects of different cytokinins with the inclusion of IMA in the culture medium. Similar to the current findings, addition of IMA to the culture medium resulted in a significant improvement on the efficacy of TDZ in *Spathiphyllum floribundum* culture (Werbrouck and Debergh, 1996). This enhancing effect of IMA was attributed to its impact upon the general mechanism of cytokinin action, i.e. its effect on changing the metabolism of exogenously applied cytokinins. Imazalil was also postulated to inhibit the activities of cytokinin degrading enzymes (Werbrouck and Debergh, 1995; 1997).

Table 19 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media using TDZ in combination with IMA (Mean ± SE).

TDZ (mg l^{-1})	IMA (mg l^{-1})	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
0	0	2.56i ± 0.24	6.22a ± 0.55	7.66a ± 0.28	7.22a ± 0.36	88.84b ± 2.32
0	2	3.06hi ± 0.26	5.12a ± 0.54	7.74a ± 0.38	6.99a ± 0.75	78.64c ± 2.90
0	4	3.50hi ± 0.29	5.67a ± 0.34	7.61a ± 0.16	6.97a ± 0.46	76.31c ± 3.09
0	8	2.72hi ± 0.16	6.83a ± 0.73	7.75a ± 0.26	6.51a ± 0.65	74.02cd ± 3.14
0.25	0	3.90ghi ± 0.12	1.35b ± 0.27	3.71b ± 0.22	2.43b ± 0.47	90.69b ± 1.35
0.25	2	7.90bc ± 0.72	1.60b ± 0.29	2.68c ± 0.15	1.62bc ± 0.31	91.26b ± 1.62
0.25	4	6.25de ± 0.35	1.60b ± 0.40	2.18cde ± 0.18	1.18c ± 0.24	66.16ef ± 2.45
0.25	8	5.44ef ± 0.74	1.07b ± 0.21	1.87def ± 0.18	0.86c ± 0.18	46.48g ± 4.52
0.5	0	5.12efg ± 0.35	1.19b ± 0.30	2.18cde ± 0.16	1.76bc ± 0.46	94.12b ± 2.71
0.5	2	10.25a ± 0.63	1.20b ± 0.22	2.29dc ± 0.16	1.52bc ± 0.28	104.18a ± 3.32
0.5	4	8.70b ± 0.75	1.30b ± 0.29	2.09cde ± 0.15	1.08c ± 0.17	91.19b ± 2.54
0.5	8	6.39de ± 0.52	0.89b ± 0.27	1.61efg ± 0.13	0.78c ± 0.22	59.48f ± 2.80
0.75	0	5.33efg ± 0.11	1.33b ± 0.32	1.66efg ± 0.09	0.89c ± 0.16	88.71b ± 2.09
0.75	2	7.22cd ± 0.68	1.00b ± 0.16	1.30fg ± 0.11	0.58c ± 0.08	89.96b ± 2.49
0.75	4	5.55ef ± 0.37	0.85b ± 0.15	1.12g ± 0.15	0.68c ± 0.15	67.68de ± 1.59
0.75	8	4.25fgh ± 0.59	0.0c ± 0.0	1.06g ± 0.16	0.0d ± 0.0	42.96g ± 2.01

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

Furthermore, the enhancement of shoot proliferation from IMA supplemented medium was recently stated to be due to its inhibitory effect upon GA biosynthesis (Werbrouck and Debergh, 1997). As reviewed by Fletcher *et al.* (2000), certain azole compounds do interfere with the biosynthesis of gibberellins (GA) and influence the morphogenesis of plants, indicating their possible use as plant growth regulators. Likewise, the imadizol fungicide, IMA, shares a similar structural feature with triazoles, i.e. a hetrocyclic ring containing a sp^2-hybridized nitrogen with a lone electron pair, which is a common feature of molecules that can inhibit ergosterol biosynthesis in fungi and can block GA synthesis in plants (Werbrouck and Debergh, 1996). This impact of the triazole chemicals on the plant's GA level was believed to cause the subsequent enhancement of endogenous cytokinins in the plant tissue (Fletcher *et al.*, 2000).

However, in all cases, IMA did not show any cytokinin effect on medium devoid of exogenous cytokinins (Werbrouck and Debergh, 1996), a finding that was also observed in the current results. Though the effect was not pronounced in krawan, korarima shoots produced on medium added with IMA and TDZ were of highly dwarfed types (Fig. 3a and 4a), requiring at least two subcultures on a PGR-free medium before attaining normal growth. However, in both species, the PGR-free medium produced well-elongated shoots with a number of functional roots (Fig. 3b and 4b).

No bipolar structures were observed from the histological study of the tiny mass of korarima buds produced on a modified MS medium supplemented with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA. The growing buds were characterized with a unipolar structure indicating their origin to be oraganogenesis rather than embryogenesis (Fig. 5).

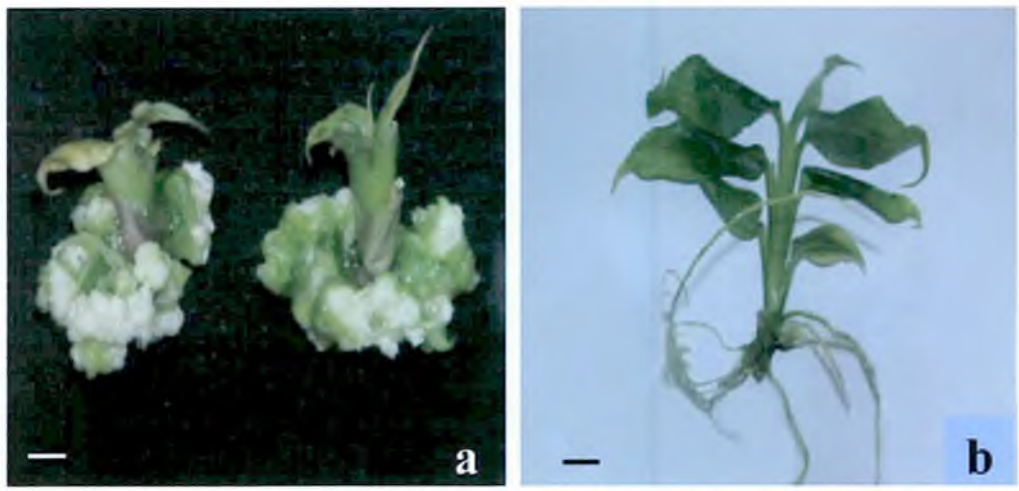

Figure 3 Korarima plantlets after eight weeks of culture on (a) modified MS medium with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA (b) PGR-free medium (bar = 1cm).

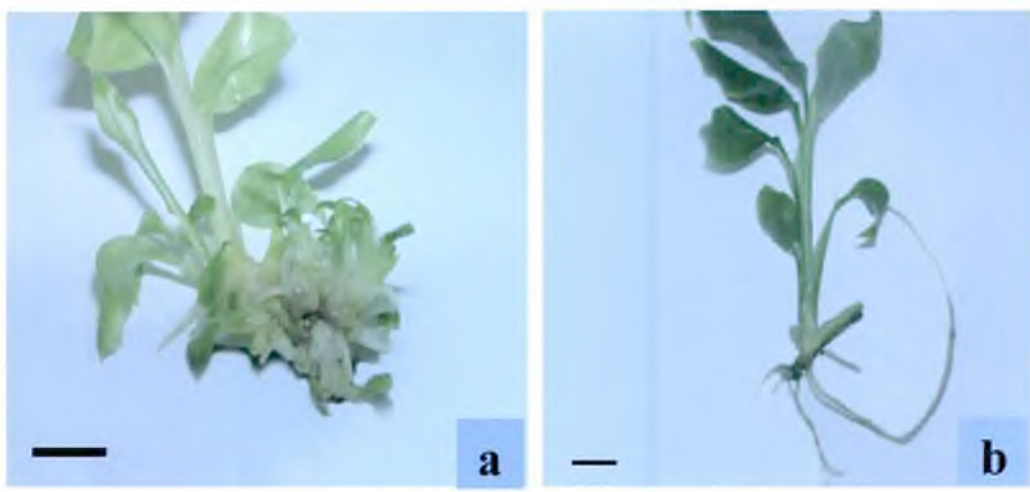

Figure 4 Krawan plantlets after eight weeks of culture on (a) modified MS medium with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA (b) PGR-free medium (bar = 1cm).

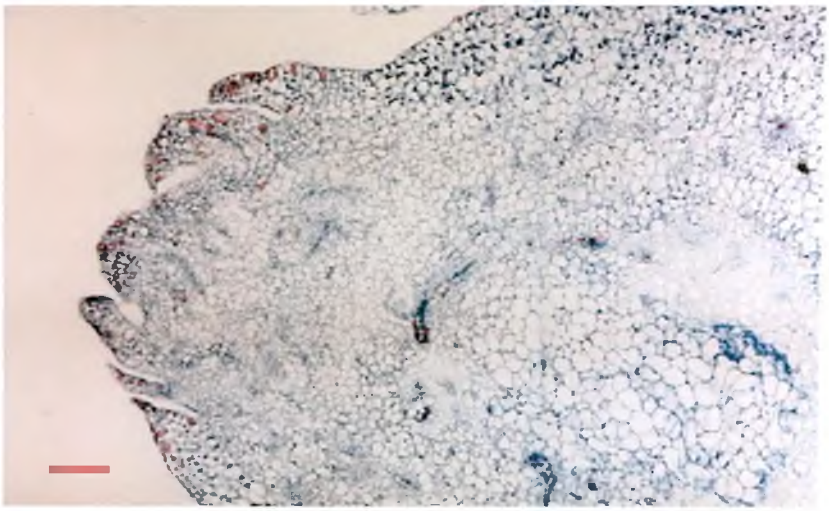

Figure 5 A histological section of korarima shoot from a modified MS medium supplemented with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA (bar = 10μ).

3.2.3. Effects of TDZ in combination with PBZ

Inclusion of TDZ into the culture medium had a significant positive effect on shoot number and dry weights of korarima. TDZ at 0.5 mg l^{-1} gave the highest number of shoots and dry weight values. However, root number, as well as root and shoot length of plantlets were highly reduced when the explants were exposed to TDZ. Addition of PBZ to the culture medium had exerted a considerable effect on all the five parameters evaluated. Accordingly, paclobutrazol at 3.0 mg l^{-1} gave the highest number of shoots and dry weight of korarima plantlets. Shoot number and dry weight of korarima plantlets had a quadratic relationship with the concentration of either TDZ and/or PBZ. All the other parameters, i.e. root number, as well as shoot and root lengths were consistently reduced with increasing concentrations of PBZ. Therefore, root number, as well as shoot and root length showed a negative linear relationship with each of the phytoregulators used in this study (Table 20).

Table 20 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media with TDZ and PBZ.

PGR	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
TDZ (mg l^{-1})					
0	2.86c	9.83a	5.27a	5.63a	87.04c
0.25	16.48b	0.59b	1.29b	0.56b	97.40b
0.5	19.42a	0.40b	0.95c	0.38b	112.85a
0.75	16.67b	0.53b	0.77c	0.32b	80.04d
PBZ (mg l^{-1})					
0	9.62c	3.46a	3.18a	2.46a	91.47b
1.5	13.41b	2.96b	2.01b	1.65b	101.02a
3.0	18.75a	2.53bc	1.60c	1.42bc	102.76a
4.5	13.90b	2.13c	1.36d	1.21c	81.29c
Prob.					
TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
BA	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ X PBZ	0.0001	0.1800	0.0001	0.0077	0.0004
% CV	22.30	33.27	31.53	27.54	17.33
Contrast					
TDZ Lin.	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ Quad.	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ Cubic	0.0001	0.0001	0.0001	0.0001	0.0001
PBZ Lin.	0.0001	0.0001	0.0001	0.0001	0.0002
PBZ Quad.	0.0001	0.2423	0.0001	0.0172	0.0001
PBZ Cubic	0.0001	0.0106	0.0001	0.0003	0.6717

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

The combined use of TDZ and PBZ in the culture medium exerted a significant synergistic effect on all the four growth and development parameters of korarima, except root number. The highest shoot number (25.94 shoots/explant) was obtained when 0.5 mg l^{-1} TDZ was combined with 3 mg l^{-1} PBZ. The use of 0.25 and 0.75 mg l^{-1} TDZ also gave relatively higher number of shoots when combined with 3 mg l^{-1} PBZ (22.63 and 22.35 shoots/explant, respectively). In the case of dry weight, the highest values were obtained when 0.5 mg l^{-1} TDZ was used in combination with either 3 mg l^{-1} or 1.5 mg l^{-1} PBZ (133.16 and 125.45 mg/explant, respectively). However, a significant reduction in root number, as well as shoot and root length of korarima were observed with the inclusion of TDZ in the culture medium (Table 21).

Unlike those produced from the aforementioned two experiments involving TDZ and BA, or TDZ and IMA, korarima shoots obtained from the PBZ supplemented TDZ medium were very minute in size (Fig. 6a). Among others, mean length of korarima shoots was reduced to less than half a centimeter when the highest levels of TDZ (0.75 mg l^{-1}) and PBZ (4.5 mg l^{-1}) were used in the culture medium (Table 21). On the other hand, when these shoots were repeatedly cultured on this same PBZ supplemented TDZ medium, they produced a mass of dome like structure covered with minute buds (Fig. 7). The microscopic observations of these tiny mass of korarima shoots produced on the medium supplemented with TDZ and PBZ revealed their origin to be organogenesis, due to the absence of any bipolar structures (Fig. 8).

The tiny korarima shoots obtained from this experiment required more than three successive sub cultures on a PGR-free medium to revive and attain normal growth. Similar to the above two experiments; the highest number of roots, as well as the longest shoots and roots in this experiment were recorded from the PGR-free medium (Table 21 and Fig. 6b).

Table 21 *In vitro* growth and development of korarima after eight weeks of culture on modified MS media using TDZ in combination with PBZ (Mean ± SE).

TDZ (mg l^{-1})	PBZ (mg l^{-1})	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
0	0	2.06i ± 0.36	10.99a ± 0.98	7.79a ± 0.31	7.58a ± 0.48	86.62de ± 5.82
0	1.5	3.17i ± 0.24	10.50a ± 1.26	4.79b ± 0.33	5.05b ± 0.50	91.36cde ± 1.08
0	3.0	3.44i ± 0.17	9.56ab ± 0.91	4.53b ± 0.14	5.18b± 0.38	88.60de ± 2.40
0	4.5	2.76i ± 0.20	8.18b ± 0.92	3.91c ± 0.16	4.64b ± 0.36	81.26e ± 4.98
0.25	0	10.94h ± 0.80	1.59c ± 0.23	2.15d ± 0.08	1.21c ± 0.15	96.25bcd ± 2.26
0.25	1.5	15.42ef ± 0.78	0.42de ± 0.14	1.72e ± 0.16	0.88cde ± 0.28	101.68bc ± 7.22
0.25	3.0	22.63b ± 0.96	0.32de ± 0.15	0.71g ± 0.10	0.12f ± 0.06	106.76b ± 3.88
0.25	4.5	16.33de ± 0.66	0.11e ± 0.08	0.64g ± 0.06	0.09f ± 0.06	84.08de ± 3.44
0.5	0	13.58fg ± 0.99	0.95cd ± 0.30	1.67e ± 0.18	0.78cd ± 0.16	102.41bc ± 2.70
0.5	1.5	18.33cd ± 1.08	0.33de ± 0.16	0.84fg ± 0.10	0.40def ± 0.19	125.45a ± 2.08
0.5	3.0	25.94a ± 0.63	0.22de ± 0.10	0.63g ± 0.06	0.28ef ± 0.14	133.16a ± 2.88
0.5	4.5	20.17c ± 0.62	0.06e ± 0.06	0.62g ± 0.03	0.04f ± 0.04	90.96cde ± 2.83
0.75	0	11.63gh ± 0.80	0.53de ± 0.18	1.24f ± 0.08	0.42def ± 0.12	80.84e ± 4.59
0.75	1.5	16.61de ± 1.00	0.72de ± 0.34	0.72g ± 0.07	0.32def ± 0.15	85.56de ± 5.01
0.75	3.0	22.35b ± 0.77	0.40de ± 0.17	0.66g ± 0.06	0.30def ± 0.11	84.36de ± 2.43
0.75	4.5	15.72e ± 0.50	0.50de ± 0.22	0.43g ± 0.04	0.26ef ± 0.11	68.87f ± 2.81

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

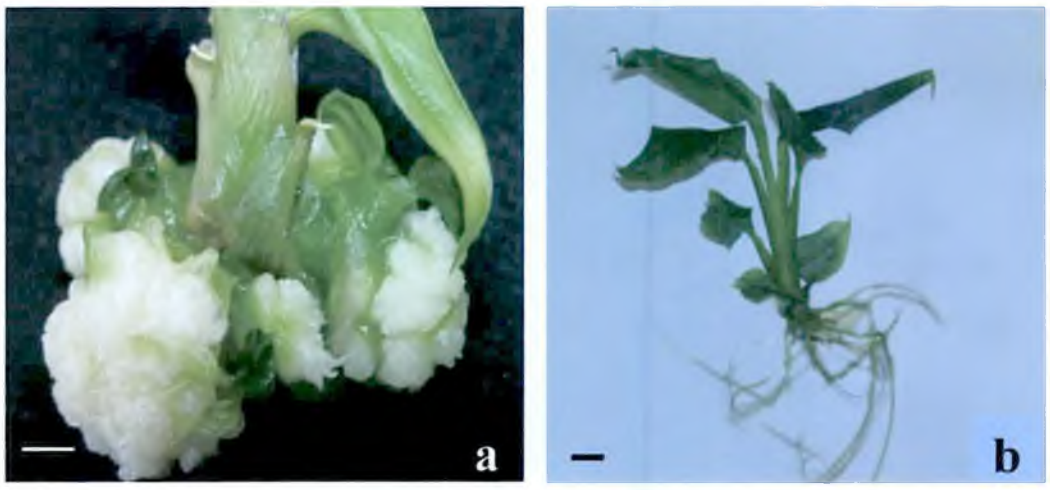

Figure 6 Korarima plantlets after eight weeks of culture on (a) modified MS medium with 0.5 mg l^{-1} TDZ and 3 mg l^{-1} PBZ (b) PGR-free medium (bar = 1cm).

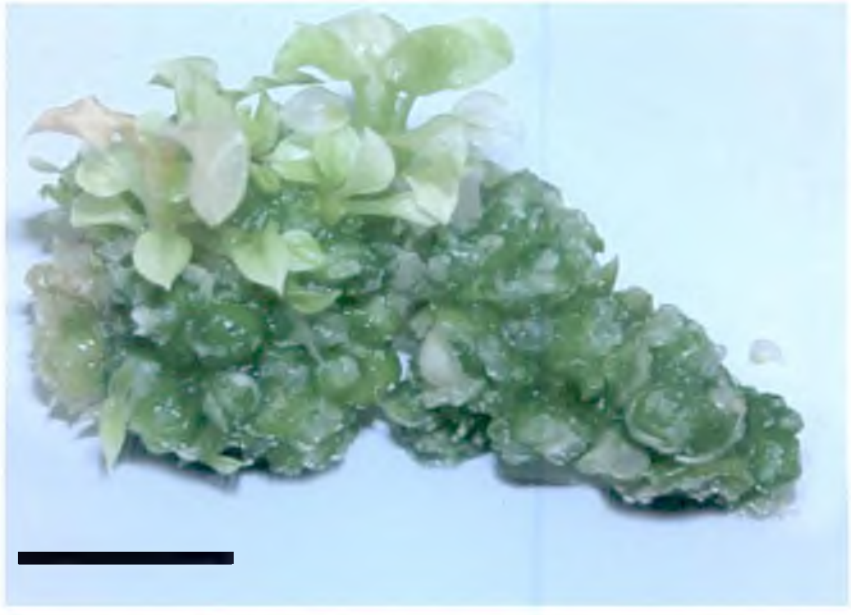

Figure 7 Korarima shoots and buds from long-term cultures on a modified MS medium supplemented with 0.5 mg l^{-1} TDZ and 3 mg l^{-1} PBZ (bar = 1cm).

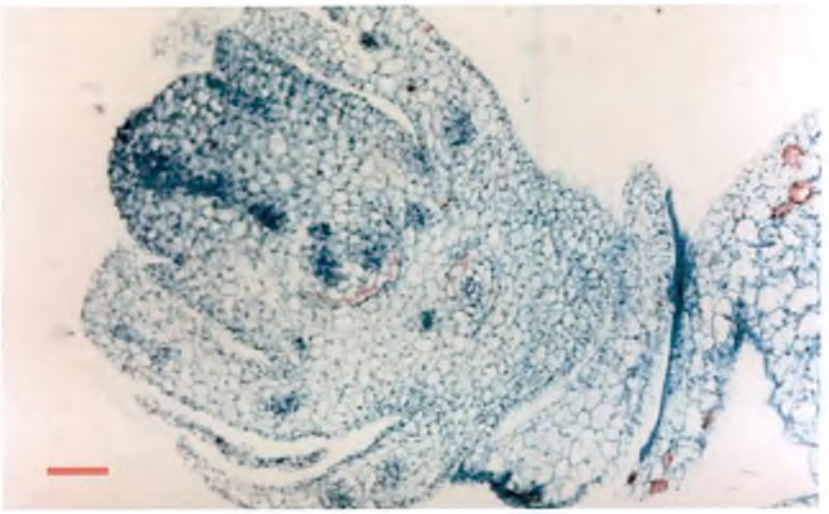

Figure 8 A histological section of korarima shoot from a modified MS medium added with 0.5 mg l^{-1} TDZ and 3 mg l^{-1} PBZ (bar = 10μ).

In line with the current findings of korarima, Lee (2001) has reported enhanced proliferation of banana shoots using TDZ in combination with PBZ, as compared to the use of the former alone. Werbrouck and Debergh (1996) also reported PBZ to enhance the activities of BA in relation to shoot multiplication. In the present study, the sole use of PBZ in the culture medium didn't affect shoot proliferation in korarima. This finding was in direct agreement with the report of Werbrouck and Debergh (1996) that stated lack of any cytokinin effect from the sole use of PBZ in *Spathiphyllum floribundum* culture.

A strong synergistic effect on shoot length was also observed in *Spathiphyllum floribundum* culture when the medium was supplemented with PBZ together with BA (Werbrouck and Debergh, 1996). As reviewed by Davis *et al.* (1988), the most obvious plant growth response to triazole treatment was reduced stem elongation and hence reduced height, due to a reduction of internode length. This was also the case in the present study. Being one of the group of chemicals collectively called triazoles, all these and similar other effects induced by PBZ could be ascribed to the increased cytokinin levels in the plant system (Fletcher *et al.*, 2000). The chemicals in the triazole group are generally known to inhibit GA biosynthesis. Thus, it is such

modulation of GA level in the plant that was believed to bring about the consequent events, including reduction of plant height (Fletcher *et al.*, 2000; Senaratna *et al.*, 2002).

On the other hand, the use of TDZ in the culture medium enhanced production of shoots in krawan, but reduced all the other four parameters (root number, shoot length, root length and dry weight of plantlets). Therefore, except shoot number that showed a quadratic trend with TDZ, all the other four parameters considered in this study of krawan revealed negative linear relationships. However, the sole use of PBZ in the culture medium was toxic for shoot proliferation and growth of krawan. Thus, the chemical had a negative relationship with all the growth and development parameters considered in this study (Table 22).

In contrast to that of korarima, the combined use of these two plant growth regulators showed an antagonistic effect on krawan shoot number. All levels of PBZ evaluated in the present experiment were toxic for krawan and resulted in death of shoots starting from the 20^{th} day of culture (Fig. 9a), whilst shoots cultured on the PGR-free medium grew normal (Fig. 9b). Relatively higher numbers of shoots (5.89 and 5.50/ explant) were obtained from the media solely supplemented with 0.5 mg l^{-1} or 0.75 mg l^{-1} TDZ, respectively (Table 23). After their initial culture on PBZ supplemented medium for only 2 or 3 weeks, transfer of treated krawan shoots to a BA containing or a PGR-free basal medium resulted in the production of faciated shoots, bearing deformed leaves and abundant thickened roots (Fig. 10).

No significant differences in number or length of krawan roots were observed with the use of different concentrations of TDZ in combination with PBZ. The general decrease in shoot length and dry weight of plantlets observed in cultures supplemented with TDZ and PBZ further substantiates the concept of antagonism resulting from the combined use of these two bioregulators in krawan. In general, it was from the PGR-free medium that plants with the highest number of roots (6.28/

explant) and dry weight values (78.17 mg/explant), as well as the longest shoots (7.20 cm) and roots (6.60 cm) were recorded (Table 23).

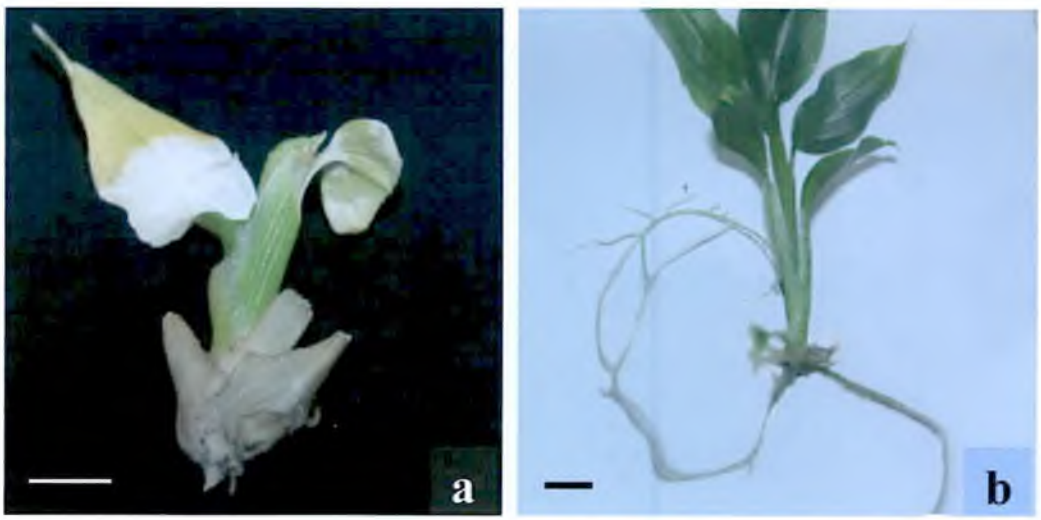

Figure 9 Krawan plantlets after eight weeks of culture on (a) modified MS medium with 1.5 mg l^{-1} PBZ and 0.5 mg l^{-1} TDZ (b) PGR-free medium (bar = 1cm).

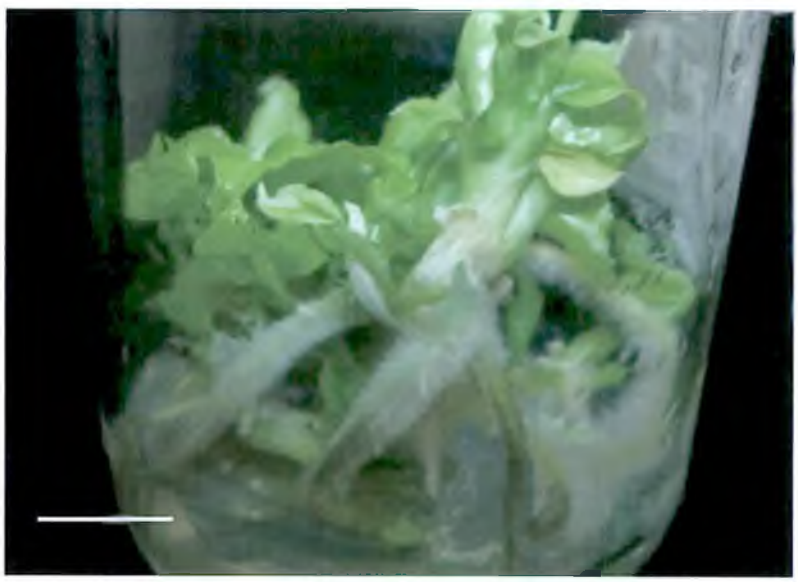

Figure 10 Krawan plantlets from PBZ supplemented TDZ medium after transfer to a BA containing medium (bar = 1cm).

Table 22 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media with TDZ and PBZ.

PGR	Shoot no.	Root no. [1/]	Shoot lg. (cm)	Root lg. (cm) [1/]	DW (mg)
TDZ (mg l^{-1})					
0	2.06c	4.16a	3.64a	4.27a	51.10a
0.25	2.46b	0.44b	2.57b	0.64b	43.09b
0.5	2.71a	0.19b	2.08c	0.25c	40.81b
0.75	2.32b	0.14b	1.60d	0.23c	34.63c
PBZ (mg l^{-1})					
0	4.77a	1.85a	3.79a	2.21a	58.47a
1.5	1.75b	1.30b	2.22b	1.31b	38.90b
3.0	1.60bc	1.03b	1.91c	1.11b	36.55b
4.5	1.41c	0.56c	1.89c	0.57c	34.95b
Prob.					
TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
PBZ	0.0001	0.0001	0.0001	0.0001	0.0001
TDZ X PBZ	0.0001	0.0001	0.0001	0.0005	0.0084
% CV	29.65	45.00	30.62	45.14	38.44
Contrast					
TDZ Lin.	0.1175	0.0001	0.0001	0.0001	0.0001
TDZ Quad.	0.0001	0.0001	0.0026	0.0001	0.6964
TDZ Cubic	0.0021	0.0001	0.0001	0.0001	0.0095
PBZ Lin.	0.0001	0.0001	0.0001	0.0001	0.0001
PBZ Quad.	0.0001	0.8767	0.0001	0.3501	0.0001
PBZ Cubic	0.0001	0.0243	0.0001	0.0353	0.0005

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

Table 23 *In vitro* growth and development of krawan after eight weeks of culture on modified MS media using TDZ in combination with PBZ (Mean ± SE).

TDZ (mg l^{-1})	PBZ (mg l^{-1})	Shoot no.	Root no. [1/]	Shoot lg. (cm) [1/]	Root lg. (cm)	DW (mg)
0	0	2.89c ± 0.20	6.28a ± 0.55	7.20a ± 0.50	6.60a ± 0.42	78.17a ± 8.82
0	1.5	2.06d ± 0.15	4.28b ± 0.84	2.59c ± 0.07	4.31b ± 0.84	43.65cd ± 3.83
0	3.0	1.70def ± 0.11	3.76b ± 0.56	2.58c ± 0.05	3.86b ± 0.46	44.09cd ± 3.14
0	4.5	1.50efg ± 0.16	2.06c ± 0.63	1.96def ± 0.16	2.03c ± 0.54	36.49de ± 5.61
0.25	0	4.79b ± 0.29	0.68d ± 0.19	3.29b ± 0.05	1.44c ± 0.36	60.64b ± 3.56
0.25	1.5	1.78de ± 0.15	0.56d ± 0.20	2.57c ± 0.22	0.36d ± 0.14	40.80cde ± 2.97
0.25	3.0	1.53defg ± 0.14	0.32d ± 0.11	1.99de ± 0.11	0.43d ± 0.16	35.13de ± 2.65
0.25	4.5	1.67def ± 0.14	0.22d ± 0.10	2.42cd ± 0.04	0.30d ± 0.16	35.23de ± 2.88
0.5	0	5.89a ± 0.24	0.33d ± 0.14	2.28cd ± 0.10	0.48d ± 0.20	51.43bc ± 4.34
0.5	1.5	1.74def ± 0.13	0.21d ± 0.10	2.28cd ± 0.20	0.31d ± 0.19	37.49de ± 3.06
0.5	3.0	2.06d ± 0.15	0.11d ± 0.08	1.67efg ± 0.11	0.16d ± 0.14	34.99de ± 2.94
0.5	4.5	1.22fg ± 0.10	0.11d ± 0.08	2.08ecd ± 0.14	0.07d ± 0.05	39.50cde ± 2.64
0.75	0	5.50a ± 0.23	0.17d ± 0.09	2.40cd ± 0.23	0.38d ± 0.21	43.53cd ± 3.27
0.75	1.5	1.44efg ± 0.12	0.22d ± 0.10	1.44fg ± 0.09	0.30d ± 0.14	33.73de ± 1.78
0.75	3.0	1.11g ± 0.08	0.11d ± 0.08	1.43fg ± 0.09	0.18d ± 0.12	32.47de ± 2.34
0.75	4.5	1.26efg ± 0.10	0.05d ± 0.05	1.14g ± 0.08	0.06d ± 0.04	29.08e ± 2.39

[1/] Transformed using square root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Shoot lg. = Shoot length, Root lg. = Root length, DW =Dry weight.

The negative effect of PBZ observed in this experiment of krawan were not only in contrast to korarima, but also to the reviews of Davis *et al.* (1988) and Fletcher *et al.* (2000), which detailed the varied benefits of this bioregulator in cultured tissues, including reduced hyperhydricity, increased cytokinin effects, etc.

The results obtained in the present study indicated even the lowest concentration of PBZ used in this study (1.5 mg l^{-1}) to be supraoptimal for growth and development of krawan. This antagonistic effect could be associated with the sensitiveness of krawan to the chemical. This could again be ascribed to the genotypic difference of krawan from the other species so far studied. This species-specific sensitivity may also be related to the endogenous GA biosynthesis.

4. Effects of Casein Hydrolysate, 2,4-D and BA on Callus Induction

Casein hydrolysate (CH), BA and 2,4-D individually exerted significant effects on callus induction of korarima. The inclusion of casein hydrolysate had also significantly improved the callus induction effect of 2,4-D (Table 24). Regarding percentage of callus produced, no significant differences were observed among the media using different concentrations of BA and/ or 2,4-D. Thus, 0.1 mg l^{-1} BA and 1 mg l^{-1} 2,4-D seem to be the best alternative for inducing friable callus in korarima (Table 25). However, a significant interaction was observed between casein hydrolysate and 2,4-D for callus induction, leading to the highest callus induction percentage of over 80% with the use of casein hydrolysate in the culture medium. Thus, the medium with 0.1 mg l^{-1} BA, 1 mg l^{-1} 2,4-D and 1 g l^{-1} CH should be employed for callus induction in korarima (Table 26).

Table 24 Callus induction of korarima and krawan after 12 weeks of culture on modified MS media with CH, BA and 2,4-D.

Addendum	Concentration	% Callus induction [1/]	
		Korarima	Krawan
CH (g l^{-1})	0	26.32b	36.12b
	1	75.98a	42.12a
BA (mg l^{-1})	0	0.00c	0.00b
	0.1	66.89a	43.78a
	0.5	51.28b	42.22a
	2.5	51.50b	43.56a
2,4-D (mg l^{-1})	0	0.00c	0.00c
	1	60.33a	40.83b
	2	54.11b	43.11ab
	4	55.22ab	45.61a
Probability	CH	0.0001	0.0021
	BA	0.0001	0.0636
	2,4-D	0.0203	0.0421
	CH XBA	0.0812	0.0001
	CH X 2,4-D	0.0325	0.7125
	BA X 2,4-D	0.0001	0.0001
	CH X BA X 2,4-D	0.0887	0.3514
% CV		10.71	8.01

[1/] Transformed using arcsine transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

In krawan, CH and 2,4-D had individually showed a significant effect on callus induction. Thus, addition of casein hydrolysate to the culture medium proved best for inducing friable callus in krawan. However, no significant effects were observed among the different levels of BA used in the present experiment. With regard to 2,4-D, no significant differences were also obtained from the use of either 2 or 4 mg l^{-1}, though the later gave a slightly higher callus percentage (Table 24). The combined use of BA and 2,4-D was effective in callus induction of krawan. The

medium supplemented with 0.1 mg l^{-1} BA and 4 mg l^{-1} 2,4-D produced the highest callus induction percentage (Table 25). A highly significant interaction was observed between casein hydrolysate and BA. The highest callus percentage was obtained from the medium supplemented with 1 g l^{-1} casein hydrolysate together with 0.1 mg l^{-1} BA (Table 27). Therefore, the medium with 1 g l^{-1} casein hydrolysate, 0.1 mg l^{-1} BA and 4 mg l^{-1} 2,4-D should be used to gain the highest percentage of callus induction in krawan.

Table 25 Callus induction of korarima and krawan after 12 weeks of culture on modified MS media with BA and 2,4-D (Mean ± SE).

PGR (mg l^{-1})		% Callus induction [1/]	
BA	**2,4-D**	**Korarima**	**Krawan**
0	0	0.00b ± 0.00	0.00d ± 0.00
0.1	1	64.67a ± 11.43	41.67bc ± 1.89
0.1	2	64.67a ± 11.22	36.33c ± 4.84
0.1	4	71.33a ± 10.14	53.33a ± 3.21
0.5	1	47.17a ± 12.61	36.67c ± 3.18
0.5	2	52.50a ± 14.65	45.00abc ± 3.42
0.5	4	54.17a ± 15.46	45.00abc ± 3.27
2.5	1	69.17a ± 8.98	44.17abc ± 2.70
2.5	2	45.17a ± 15.80	48.00abc ± 3.06
2.5	4	40.17a ± 13.40	38.50ab ± 1.31

[1/] Transformed using arcsine transformation before analysis.
Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

These findings related to the beneficial effects of CH for callus induction in both korarima and krawan were in direct agreement with the report of Srinivasa Rao *et al.* (1982), who stated the benefits of using 1 mg l^{-1} casein hydrolysate, together with 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA and 18% CW for the production of highly proliferating calli in cardamom.

Table 26 Callus induction of korarima after 12 weeks of culture on modified MS media with casein hydrolysate and 2,4-D (Mean ± SE).

Addendum		% Callus induction [1/]
CH (g l^{-1})	2,4-D (mg l^{-1})	
0	0	0.00c ± 0.00
0	1	36.67b ± 5.07
0	2	23.44b ± 4.64
0	4	26.78b ± 6.40
1	0	0.00c ± 0.00
1	1	84.00a ± 3.33
1	2	84.78a ± 2.35
1	4	83.67a ± 3.81

[1/] Transformed using arcsine transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

The combined use of 2,4-D and BA for callus induction had been reported for numerous plant species. In several species, calli were effectively induced on medium supplemented with 0.5 - 2 mg l^{-1} 2,4-D in combination with 0.1 - 1.5 mg l^{-1} BA (Deeks *et al.*, 2001; Jin *et al.*, 2002; Kumar *et al.*, 2002; Zhao *et al.*, 2003). Among members of the family Zingiberaceae, Malamug *et al.* (1991) obtained the highest degree of embryonic calli from ginger shoot tips cultured on the medium supplemented with 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. In *Kaempferia galanga*, Vincent *et al.* (1991) also observed proliferation of white friable calli from explants cultured on MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. Similarly, Salvi *et al.* (2000) also induced the highest number of calli from turmeric inflorescence cultured on MS medium added with 5 mg l^{-1} BA and 0.2 mg l^{-1} 2,4-D.

In both korarima and krawan, the calli produced using these formulations were friable, creamy, and white in color (Fig. 11a and 11b). The current finding regarding the nature and regeneration capacity of calli was in direct agreement with the reports

of Bajaj *et al.* (1993). From their experiment on cardamom, these authors had reported the use of 2,4-D to result in profuse, white and friable callus, which did not regenerate on subsequent efforts. However, in most cases, calli were readily turned green when transferred to the medium containing 0.5 mg l^{-1} TDZ added with 2 mg l^{-1} IMA (Fig. 12a and b), or 3 mg l^{-1} BA and 1 mg l^{-1} kinetin. However, no plantlets were regenerated from the calli produced in both genera. Transfer to a PGR-free medium added with 2 g l^{-1} activated charcoal led to the death of calli.

Table 27 Callus induction of krawan after 12 weeks of culture on modified MS media with casein hydrolysate and BA (Mean ± SE).

Addendum		% Callus induction[1/]
CH (g l^{-1})	BA (mg l^{-1})	
0	0	0.00c ± 0.00
0	0.1	37.56b ± 3.33
0	0.5	36.67b ± 2.24
0	2.5	45.33ab ± 2.11
1	0	0.00c ± 0.00
1	0.1	50.00a ± 2.71
1	0.5	47.78a ± 2.15
1	2.5	41.78ab ± 2.49

[1/] Transformed using arcsine root transformation before analysis.

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

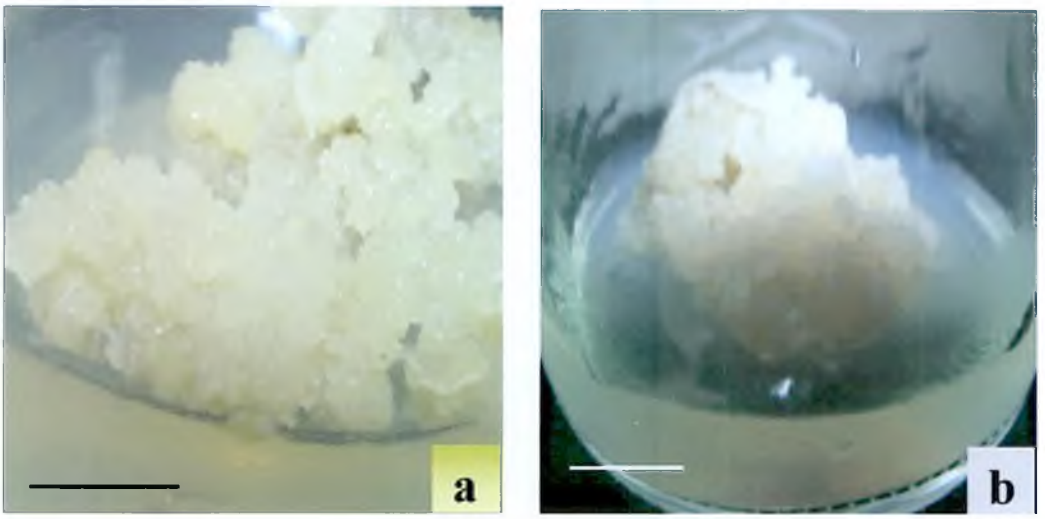

Figure 11 Callus induction on a modified MS medium supplemented with BA, 2,4-D and casein hydrolysate (a) korarima (b) krawan (bar = 1cm).

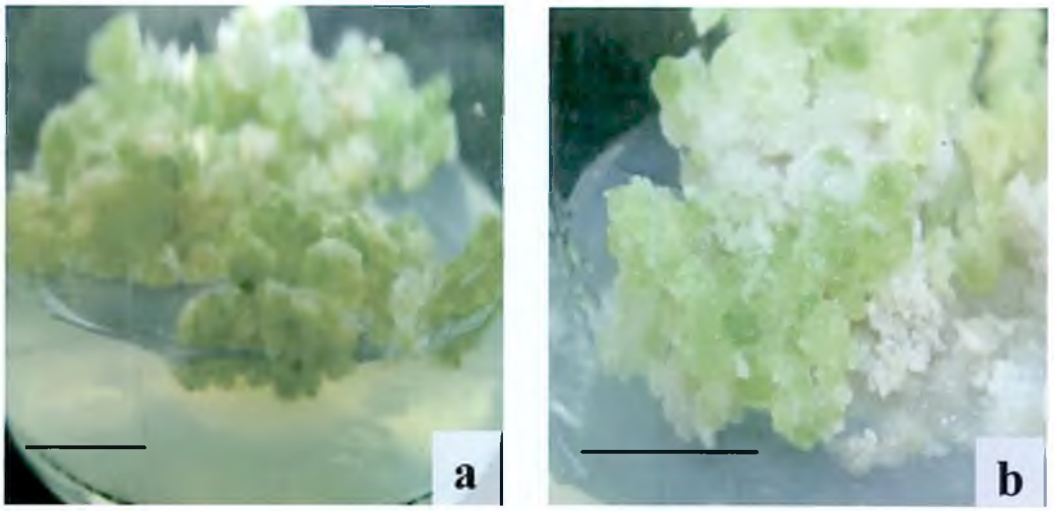

Figure 12 Calli after transfer to a modified MS medium added with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA (a) korarima (b) krawan (bar = 1cm).

5. *In Vitro* Polyploid Induction

5.1. Survival of treated shoots

The survival rate of treated korarima shoots from the lowest concentration of colchicine was lower than that of oryzalin (45.83% and 74.07%, respectively). Similarly, the numbers of krawan shoots that survived the lowest dosage of colchicine and oryzalin treatments were 46.67% and 54.17%, respectively (Table 28).

Table 28 Survival percentage of korarima and krawan shoots after seven days of colchicine and oryzalin treatment.

Agent (μM)	Number and % Shoots			
	Korarima		Krawan	
	Treated	Survived	Treated	Survived
0	30	30 (100.00)	30	29 (96.67)
Colchicine				
125	30	11 (45.83)	30	14 (46.67)
250	30	8 (29.63)	30	8 (33.33)
375	30	7 (23.33)	30	7 (23.33)
500	30	3 (11.54)	30	3 (12.50)
Oryzalin				
10	30	20 (74.07)	30	13 (54.17)
20	30	22 (73.33)	30	13 (43.33)
40	30	17 (62.96)	30	12 (40.00)
60	30	12 (44.44)	30	11 (36.67)
80	30	10 (37.04)	30	8 (29.63)

5.2. Screening of polyploid plants

5.2.1. Morphological characters

Based on their morphological differences from the diploid control, the highest percentage and number of putative polyploid plants from the colchicine and oryzalin treatments of korarima were 11 and 16 plants, respectively (Table 29). Similarly, a total of 4 plants from the colchicine and 24 from the oryzalin treatments of krawan exhibited detectable differences in their morphological characters (Table 30). In both species, the main morphological characters used for preliminary screening of the putative polyploid plants were robustness of the plantlets, as well as color intensity and size of the leaves, using the diploid controls as a baseline.

Table 29 Effects of colchicine and oryzalin on the *in vitro* polyploid induction of korarima.

Agent (µM)	No. of Shoots tested for polyploidy	Number and percent of putative polyploid shoots from each group based on						
		Morphology	Stomata			Ploidy[1/]		
			No.	LG	Index	DP	TP	MP
0	5	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	5	0	0
Colchicine								
125	11	5 (45.45)	5 (45.45)	5 (45.45)	5 (45.45)	6 (54.54)	3 (27.27)	2 (18.18)
250	8	2 (25.00)	2 (25.00)	2 (25.00)	2 (25.00)	6 (75.00)	0 (0.00)	2 (25.00)
375	7	2 (28.57)	2 (28.57)	2 (28.57)	2 (28.57)	5 (71.43)	0 (0.00)	2 (28.57)
500	3	2 (66.67)	2 (66.67)	2 (66.67)	2 (66.67)	1 (33.33)	0 (0.00)	2 (66.67)
Oryzalin								
10	20	9 (33.33)	9 (45.00)	9 (45.00)	9 (5.00)	11 (55.00)	4 (20.0)	5 (25.00)
20	22	6 (20.00)	6 (27.27)	6 (27.27)	6 (4.54)	16 (72.73)	0 (0.00)	6 (100.00)
40	17	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	17 (100.00)	0 (0.00)	0 (0.00)
60	12	1 (3.70)	1 (8.33)	1 (8.33)	1 (8.33)	11 (91.67)	0 (0.00)	1 (8.33)
80	10	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	10 (100.00)	0 (0.00)	0 (0.00)

[1/] DP= Plantlets having diploid chromosome number; TP= Plantlets with tetraploid chromosome count; MP= Plantlets with distinct morphological difference but similar chromosome number with the diploid controls.

Table 30 Effects of colchicine and oryzalin on the *in vitro* polyploid induction of krawan.

Agent (μM)	No. of Shoots tested for polyploidy	Number and percent of putative polyploid shoots from each group based on						
		Morphology	Stomata			Ploidy[1/]		
			No.	Length	Index	DP	TP	MP
0	5	0	0	0	0	5	0	0
Colchicine								
125	14	4 (28.57)	4 (28.57)	4 (28.57)	1 (7.14)	10 (71.43)	0 (0.00)	4 (28.57)
250	8	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	8 (100.00)	0 (0.00)	0 (0.00)
375	7	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	7 (100.00)	0 (0.00)	0 (0.00)
500	3	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	3 (100.00)	0 (0.00)	0 (0.00)
Oryzalin								
10	13	5 (38.46)	3 (23.08)	5 (38.46)	0 (0.00)	8 (61.54)	0 (0.00)	5 (38.46)
20	13	9 (69.23)	6 (46.15)	9 (69.23)	0 (0.00)	4 (30.77)	0 (0.00)	9 (69.23)
40	12	8 (66.67)	5 (41.67)	7 (58.33)	0 (0.00)	4 (33.33)	0 (0.00)	8 (66.67)
60	11	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	11 (100.00)	0 (0.00)	0 (0.00)
80	8	2 (25.00)	2 (25.00)	2 (25.00)	0 (0.00)	6 (75.00)	0 (0.00)	2 (25.00)

[1/] DP= Plantlets having diploid chromosome number; TP= Plantlets with tetraploid chromosome count; MP= Plantlets with distinct morphological difference but similar chromosome number with the diploid controls.

5.2.2. Stomatal characters

All the 27 putative tetraploid korarima shoots from the morphological screening showed distinct differences from their diploid relatives based on their stomata number, length and stomatal indices (Table 29). They were distinguished with their reduced stomatal density (2.20 – 5.10/mm^2), longer stomata (14.20 - 20.60μ) and lower stomatal indices (4.75 - 7.39) than the controls that recorded 8.80 – 10.70/mm^2, 10.80 - 11.60μ and 8.81 – 13.48, for the respective parameters (Appendix Tables 13 and 14; Figs. 13a and b).

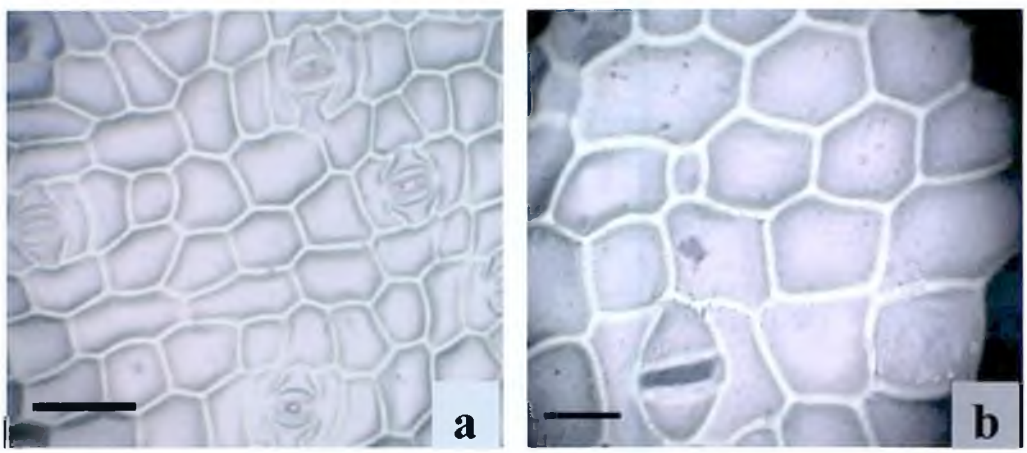

Figure 13 Stomata from the abaxial leaf surface of (a) diploid (bar = 19μ) and (b) tetraploid korarima shoots (bar = 13μ).

In krawan, a clear distinction was observed between the diploid controls and the putative tetraploid plants for these three stomatal parameters. Among these, only 4 plants from the colchicine treatment and 16 from the oryzalin treatment showed a common clear difference from their diploid counter parts based on their stomatal density and length. With regard to stomatal index, however, only 1 plant from the colchicine treatment and none from the oryzalin treatment displayed a significant difference than the diploid controls (Table 30). Compared to the controls, the potentially polyploid krawan plants had significantly fewer stomata density (8.90 – 12.10/mm^2) and longer stomata (13.10 –16.00μ) on the abaxial side of their leaves (Appendix Tables 15 and 16, Figs. 14a and b). However, the stomatal index had a

great variation within the diploid control (Appendix Table 15). Thus, it may not be a very good criterion to differentiate ploidy levels in krawan.

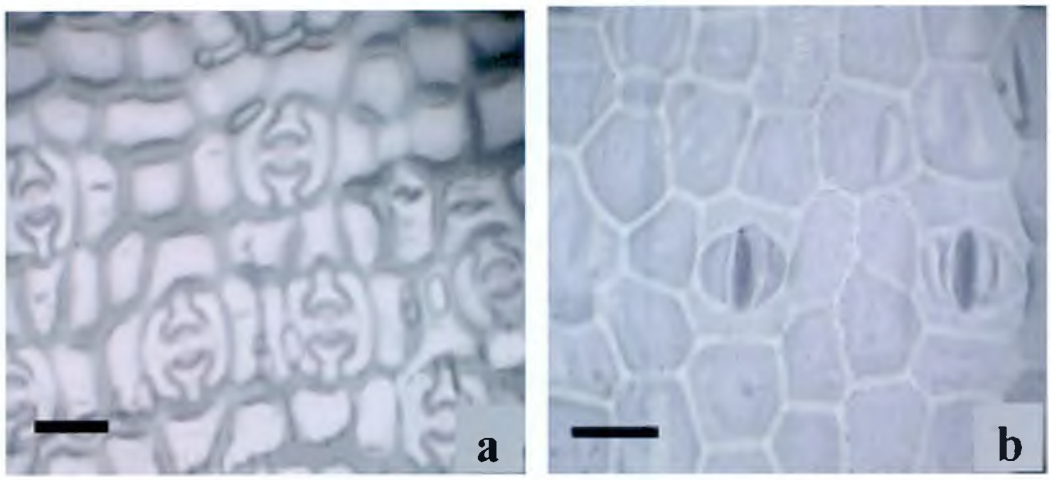

Figure 14 Stomata from the abaxial leaf surface of (a) diploid (bar = 17μ) and (b) tetraploid krawan shoots (bar = 16μ).

Similar to most other herbaceous plant species, both korarima and krawan plants were amphistomatous, bearing stomata on both sides of their leaf surfaces (Weyers and Meidner, 1990). The stomatal frequencies in both genera were also higher on the abaxial surface. The stomatal characters of both species from the different treatments generally agreed with the morphological characters. Different workers had suggested the effectiveness of different stomatal and morphological characters for preliminary selection of putative tetraploid from their diploid counterparts in several plant species (Speckmann *et al.,* 1965; Cohen and Yao, 1996). Among the varied morphological characters used for evaluation, those related to stomatal character, including stomatal density, length and index are stated to be much more efficient (Speckmann *et al.,* 1965; Cohen and Yao, 1996; Chakraborti *et al.*, 1998; Kadota and Niimi, 2002).

In banana, the stomatal size and density showed significant differences between the micropropagated diploid and tetraploid plantlets. Therefore, the tetraploids had considerably larger stomata than the diploids. Besides, the diploid

plantlets had significantly more number of stomata per unit area than the tetraploids (Hamill *et al.,* 1992; van Duren *et al.*, 1996). Of these two stomatal features, however, stomatal length measurements taken from the middle surface of any leaf were stated as a particularly useful parameter for distinguishing tetraploids from diploids under *in vitro* condition (Hamill *et al.,* 1992). In another instance, Chakraborti *et al.* (1998) had observed relatively large sized and less frequent stomata in tetraploid mulberry plants than in the diploids. Meenattoor and Nair (1993) also reported reduced number of stomata and longer stomatal guard cells as indicatives of putative tetraploids in lemongrass.

Similarly, tetraploid cardamom plants were observed to be robust, having thicker leaves, larger stomata and larger pollen grains than their diploid relatives. Besides, when stomatal index was calculated, the number of stomata in tetraploid leaves was lower than that in diploid controls (Sudharshan, 1989).

5.2.3. Chromosome count

In both species, the diploid chromosome numbers were 2x = 48. The chromosomes of korarima were very small in size (Fig. 15), than those of krawan (Fig. 16). Based on the chromosome count, none of the krawan plants were found to contain tetraploid chromosomes (Table 30). However, of the putative tetraploid korarima shoots selected using plant morphology and stomatal characters, three from the 125 µM colchicine treatment (P2), and four from the 10 µM oryzalin treatments (P6) were confirmed to be solid tetraploids (Table 29; Fig. 17). This lack of agreement between the results from the stomatal characters and the chromosome counts could be indicative for the existence of cytochimeras in the population.

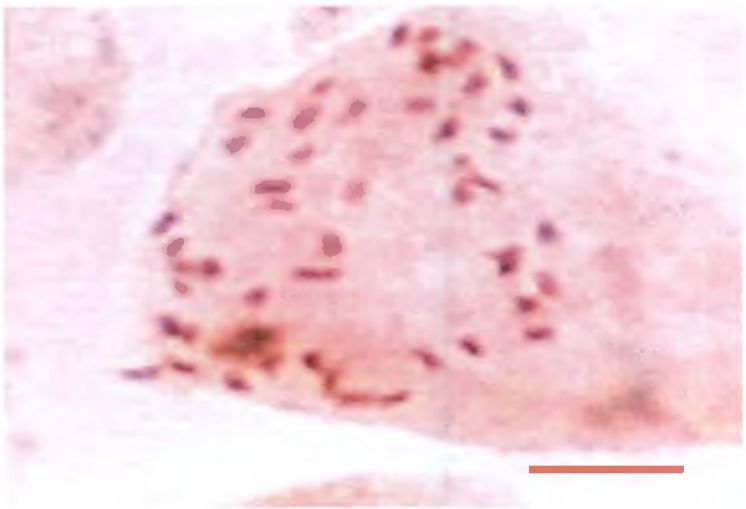

Figure 15 Mitosis chromosomes of a diploid korarima (2n = 48) (bar = 10μ).

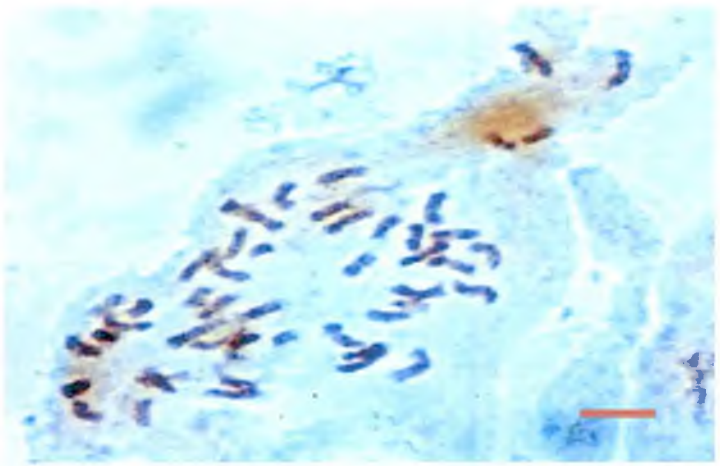

Figure 16 Mitosis chromosomes of a diploid krawan (2n = 48) (bar = 10μ).

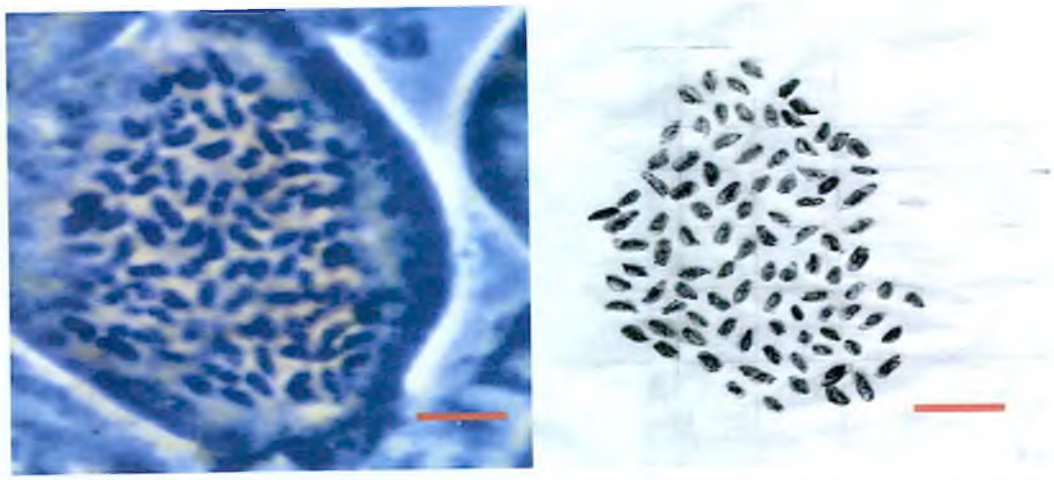

Figure 17 Mitosis chromosomes of a tetraploid korarima (2n = 4N = 96) (bar = 10μ).

SUMMARY AND CONCLUSIONS

The highest *in vitro* germination and germination rate index, as well as the shortest mean days to germination of korarima seeds were obtained from the use of a sterile cotton pad soaked with distilled water. The technique can be a foundation for embryo-rescue works of korarima in a future hybridization program.

In both korarima and krawan, the best medium for shoot growth was a modified MS added with 5% coconut water, 3 mg l^{-1} BA and 1 mg l^{-1} kinetin and 3% sucrose. High rate *in vitro* shoot proliferation of korarima could be obtained from the use of 0.5 mg l^{-1} thidiazuron in combination with 3 mg l^{-1} paclobutrazol. Shoots from the medium, however, required at least three subcultures on a PGR-free MS medium for reviving their normal growth and rooting. Continuous proliferation of shoots could also be obtained from repeated culture of explants on this same medium before transfer to the PGR-free medium for elongation and rooting. On the other hand, considerable number of high quality shoots could readily be obtained using 0.5 mg l^{-1} TDZ in combination with 3 mg l^{-1} BA. In the case of krawan, the medium with 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA gave the highest rate of shoot proliferation. In both species shoot elongation and rooting was attained with a relative ease using a PGR-free MS medium. The protocols developed from this study could pave the way for subsequent *in vitro* works of these important spices and medicinal plants of high economic potential. They could also serve as the basis for the *in vitro* preservation endeavors of these endangered species, which requires urgent intervention to cope up with the current severe genetic erosion.

In both species, friable calli could be induced from culturing shoot explants on MS medium added with 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BA and 1 g l^{-1} casein hydrolysate. Attempts to regenerate the calli from both species were unsuccessful in this study. But in both cases, calli had turned green and showed an indication of regeneration on media added with either of 0.5 mg l^{-1} TDZ and 2 mg l^{-1} IMA, or 3 mg l^{-1} BA and 1 mg

l^{-1} kinetin. For both plant species, development of effective regenerating media requires further investigation.

The diploid chromosome number of korarima and krawan were counted to be 48. *In vitro* induction of tetraploidy in korarima was attained using the lowest concentrations of both chemicals tested in the present study (i.e. 125 μM colchicine or 10 μM oryzalin solutions). The relatively less toxic nature of oryzalin makes it promising for future *in vitro* polyploid induction works of korarima. Only mixoploid krawan plants were obtained from this experiment. Further multiplication ought to be performed to isolate a solid tetraploid shoot in krawan.

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APPENDIX

Appendix Table 1 Summary of disinfestation techniques used in micropropagation of cardamom and its close relatives

Species	Explant	Primary disinfestation	Secondary disinfestation	Reference
Elettaria cardamomum	Young sprouting buds (12-20mm) with small portion of rhizome	ND	0.12% (w/v) mercuric chloride and later washed for 4-5 times	Nadgauda *et al.* (1983)
E. cardamomum	Sprouting buds (10-20mm)	ND	Immersed in 25% Chloriwat (1% chlorine) + a few drops of Tween 40 for about 45 min Rinsed three times But had some random contamination However, when ampiciline (100 mg l^{-1}) and carbendazim (30 mg l^{-1}) were added to it no contamination was observed for the initial 10 days	Vatsya *et al.* (1987)
E. cardamomum	Shoot premordium tips (3mm)	A quick dip in 95% alcohol	Treatment in 0.5% mercuric chloride for 12 min	Reghunath and Bajaj (1992)
E. cardamomum	Young vegetative buds	ND	0.1-.02% mercuric chloride	Lukose *et al.* (1993)
E. cardamomum	Juvenile shoot primoridia (20 – 30 mm) raised from *in vitro* cultured mother plants	Ethanol (95%) for 15 sec.	0.15% aqueous mercuric chloride for 1 – 2 min. (Added is one drop of Teepol/ 50 ml) Rinsed trice	Reghunath and Priyadarshan (1993)
E. cardamomum	Rhizome pieces with sprouting buds	Explants washed in running tap water	0.1% mercuric chloride solution for 7 – 10 min. Rinsed 3 – 5 times	Nirmal Babu *et al.* (1999)
Amomum subulatum	Young sprouting buds from rhizome	Washed in running tap water and treated with 0.3% copper oxychloride and Teepol for 1hr	Immersed in 0.1% mercuric chloride for 5-7 min.	Sajina *et al.* (1997)

ND= not described in the report.
In all cases rinsing was done with sterilized distilled water.

Appendix Table 2 Summary of disinfestation techniques used in micropropagation of ginger and its close relatives.

Species	Explant	Primary disinfestation	Secondary disinfestation	Reference
Zingiber officinale	Buds emerged on rhizomes were excised and a few scale leaves removed	ND	0.5% sodium hypochlorite (10% Clorox) for 10 min. Rinsed twice **But, 50% were found contaminated**	Hosoki and Sagawa (1977)
Z. officinale	Sprouting buds (pale yellow in color) taken from thoroughly washed and sprouting rhizomes	ND	0.2% aqueous solution of mercuric chloride Rinsed several times	Bhagyalakshmi and Singh (1988)
Z. officinale	Excised shoot tips from stored rhizomes	Ultrasonic cleanser in 0.1% (v/v/) positive soap (Benzalkonium Chloride: Osavan) solution for 5 min	A solution of sodium hypochlorite containing 0.5% active chlorine and a wetting agent (Tween-20) for 10 min after a brief rinse in 70% (v/v) ethanol solution Rinse three times	Inden *et al.* (1988)
Z. officinale	Sprouting buds- after planting out on sand (3 cm)	ND	0.1 % mercuric chloride for 15 min. Rinsed 5 – 6 times	Balachandran *et al.* (1990)
Z. officinale	Axillary buds from rhizomes (dissected)	ND	1% potassium hypochlorite for 20 min. Rinsed 3 times	Dekkers *et al.* (1991)
Z. officinale	New emerging buds (1 – 1.5 cm) from rhizomes sprouted in the lab (after thorough washing)	Washed in running water for 5 min. Cleansed in an ultrasonic washer and immersed in a 0.1% (v/v) detergent (Osvan) for 5 min. Rinsed with 70% ethanol	Sodium hypochlorite (0.5% active chlorine and a wetting agent (Tween 80) for 10 minutes Rinsed trice under aseptic condition	Malamug *et al.* (1991)
Z. officinale	Aerial shoots, composed of sheathing leaf bases enclosing the youngest leaf inside (cut in to 4 – 5cm long sections)	Washed in running water	0.1 % mercuric chloride solution for 5 – 10 min Rinsed 3 –4 times in sterile water	Nirmal Babu *et al.* (1992)
Z. officinale	Sprouting buds from rhizome	ND	Aqueous solution of 0.1% mercuric chloride and 2 drops of Tween 20 for 10-12 min Rinse 3-4 times But more than 40% contamination	Sharma and Singh (1997)
Z. officinale	Apical and lateral buds from sprouted rhizomes (1-3cm)	70% ethanol for 2 min	Immerse in 1% bleach + Tween Rinse three times	Devi (1999)

Appendix Table 2 (cont'd)

Species	Explant	Primary disinfestation	Secondary disinfestation	Reference
Alpina galanga	Emerging buds from stored rhizomes (trimmed)	Soaked in Tween 20 solution for 15 min.	0.2% mercuric chloride for 5 – 7 min. Rinsed trice	Borthakur *et al.* (1999)
A. purpurata	Bulbils arising in the inflorescence (dissected)	ND	1% potassium hypochlorite for 20 min. Rinsed trice	Dekkers *et al.* (1991)
A. purpurata	Axillary buds from rhizomes (dissected)	ND	1% potassium hypochlorite for 20 min. Rinsed 3 times	Dekkers *et al.* (1991)
Costus alfer	Axillary buds from rhizomes (dissected)	ND	1% potassium hypochlorite for 20 min. Rinsed 3 times	Dekkers *et al.* (1991)
C. lacanusianus	Axillary buds from rhizomes (dissected)	ND	1% potassium hypochlorite for 20 min. Rinsed 3 times	Dekkers *et al.* (1991)
Zingiber cassumunar	Shoot tips dissected from rhizomes in storage	95% ethanol for 1 min. + 30 mg l^{-1} chloramphenicol or cloxacilline or neomycin, or 15 mg l^{-1} amoxicillin	10% Clorox solution with 2 – 3 drops of Tween-20 for 30 min. Rinsed trice	Poungpet and Krisana (1993)
Kaempferia galanga	Young sprouting buds (6-8mm) excised from the rhizome	Washed thoroughly with dilute detergent solution	0.1%aqueous mercuric chloride solution for 10 min Rinsed 5-6 times	Vincent *et al.* (1991)
K. galanga	Rhizome pieces	Washed thoroughly with tap water and immersed in 70% ethanol for 2 min and washed with Cetrimide for 10 min and rinsed thoroughly	0.1% aqueous mercuric chloride solution for 6-8 min Rinsed several times	Shirin, *et al.* (2000)
Zingiber spectabile	Axillary buds	Washed with running water for 5 min	2.5% aqueous solution of sodium hypochlorite, with 1 drop of Tween (10% solution) per 100 ml and shaken for 20 min at 120 rpm.	Faria and Illg (1995)

ND= not described in the report.
In all cases rinsing was done with sterilized distilled water.

Appendix Table 3 Summary of disinfestation techniques used in micropropagation of turmeric and its close relatives.

Species	Explant	Primary disinfestation	Secondary disinfestation	Reference
Curcuma domestica	Buds excised from sprouting rhizomes	ND	A freshly prepared chlorine water	Kuruvinashetti and Iyer (1982)
C. domestica	Emerging buds taken from repeatedly washed rhizome with detergent and tap water	70% ethanol for 2 min and 0.5% sodium hypochlorite (10% Clorox) for 10 min	After removal of a few scale leaves the basal part was immersed in 2% Purelox solution for 5 min Rinsed three times	Mukhri and Yamaguchi (1986)
C. domestica	Young sprouting rhizomes buds excised from rhizomes	Washed with water containing a few drops of Teepol and again with distilled water	0.1% mercuric chloride for 15 min Rinse three times	Keshevachandran and Khader (1989)
C. domestica	Sprouting buds- after planting out on sand (3 cm)	ND	0.1 % mercuric chloride for 15 min. Rinsed 5 – 6 times	Balachandran *et al.* (1990)
C. domestica	Axillary buds from rhizomes (dissected)	ND	1% potassium hypochlorite for 20 min. Rinsed 3 times	Dekkers *et al.* (1991)
C. domestica	Buds of 2 – 5 cm long from rhizome Rinsed	70% ethanol for 30 sec.	Immersed in 3% sodium hypochlorite (with one drop/ 50ml of Tween 20) for 10 min. Rinsed trice	Sugaya (1992)
C. longa	Buds from field grown rhizomes	70% (v/v) ethyl alcohol for 30 sec	0.1% (w/v) HgCl2 solution for 15 min Rinsed with sterile water 5 - 6 times Cultured in medium added with sterile streptomycin sulphate (750 mg l^{-1}) for 4 weeks	Salvi *et al.* (2001; 2002)
C. longa	Actively growing shoots	Washed thoroughly in running tap water, and rinsed with 70% ethanol for 1 min Commercial bleach (1.5% sodium hypochlorite) and 50 µl Tween 80 for 20 min and rinsed three times	Terminal buds were treated with commercial bleach solution (0.75%) sodium hypochlorite containing 50 µl Tween 80 for 10 min, and rinsed three times.	Prathanturarug *et al.* (2003)
C. aeruginsa	Sprouting buds- after planting out on sand (3 cm)	ND	0.1 % mercuric chloride for 15 min. Rinsed 5 – 6 times	Balachandran *et al.* (1990)
C. amada	Axillary buds from rhizomes (dissected)	ND	1% potassium hypochlorite for 20 min. Rinsed 3 times	Dekkers *et al.* (1991)

Appendix Table 3 (cont'd)

Species	Explant	Primary disinfestation	Secondary disinfestation	Reference
C. caesia	Sprouting buds- after planting out on sand (3 cm)	ND	0.1 % mercuric chloride for 15 min. Rinsed 5 – 6 times	Balachandran *et al.* (1990)
C. xanthorhiza	Emerging buds taken from repeatedly washed rhizome with detergent and tap water	70% ethanol for 2 min and 0.5% sodium hypochlorite (10% Clorox) for 10 min	After removal of a few scale leaves the basal part was immersed in 2% Purelox solution for 5 min Rinsed 3 times	Mukhri and Yamaguchi (1986)
C. zedoaria	Rhizome and buds	70% ethanol for 30 sec.	10% chlorinated lime (no time specified)	Sugaya (1992)
C. alismatifolia	Young inflorescence (at the stage where all their bracts were tightly close)	Rinsed in running water for 30 min Soaked in 70% ethanol for a few min	0.5% NaOCl and shaked for 15 min Rinsed with sterilized water for 3 min	Wannakrairoj (1997)
C. alismatifolia	Field grown sprouting rhizomes were collected and dried for a week Buds were excised	Excised buds were rinsed under running water for 30 min Kept in hot water (52°C) for 5-10 min.	Dipped in 70% ethanol Disinfected in 0.79% NaOCl for 10 min Rinsed with sterilized water	Wannakrairoj (1997)

ND= not described in the report.
In all cases rinsing was done with sterilized distilled water.

Appendix Table 4 Summary of *in vitro* callus induction studies on cardamom and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
E. cardamomum	Sprout from seeds	MS + CW (18%)	2,4-D (2)	Good callusing	Srinivasa Rao *et al.* (1982)
E. cardamomum	Sprout from seeds	MS + CW (18%) +CH (1gm/l)	2,4-D (2) + BAP (0.5)	Callusing exuberant	Srinivasa Rao *et al.* (1982)
E. cardamomum	Sprout from seeds	MS + CW (18%)	IAA (1) or IBA (1) + NAA (2)	Callus grew as vascular nodules	Srinivasa Rao *et al.* (1982)
E. cardamomum	Shoot bud (20 – 50mm) pretreated with 200 mg l^{-1} K or BA *in-vivo* for 6 – 24 h	MS + CW (5%)	NAA (4) + BAP (1)	Callus, shoots	Reghunath and Bajaj (1992)
E. cardamomum	Shoot meristems	MS + CW (5%) + agar (5gm/l)	NAA (4) + BA (1)	Callus induction	Bajaj *et al.* (1993)

[1/] MS = Murashige and Skoog (1962); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; 2,4-D= 2,4-Dichlorophenoxyacetic acid; CH= casein hydrolsate.

Appendix Table 5 Summary of *in vitro* callus induction studies on ginger and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
Z. officinale	Shoot tips	Revised MS (MS major salts + RN minor elements + organic addenda + sucrose (2%) + agar (0.8%)	ND	Callus induction	Malamug *et al.* (1991)
Z. officinale	Inner most tissue of young leaves	MS	2,4-D (13.6μM)	Callus	Nirmal Babu *et al.* (1992)
Z. officinale	Young leaf segments from *in vitro* shoot cultures	MS	Dicamba (2.7μM)	Embryogenic callus induction and maintenance	Kacker *et al.* (1993)
Kaempferia galanga	Young sprouting buds (6-8mm) from rhizome	MS	2,4-D (1) + BAP (0.5)	Callus	Vincent *et al.* (1991)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 6 Summary of *in vitro* callus induction studies on turmeric and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
C. domestica	Emerging buds (2-3mm) from rhizomes	RN + 2% sucrose +0.8% agar	BA (10) + 2,4-D (1) or BA (10) + NAA (15)	Callus	Mukhri and Yamaguchi (1986)
C. domestica	Emerging buds (2-3mm) from rhizomes	RN + 2% sucrose +0.8% agar	BA (10) + NAA (15)	Callus	Mukhri and Yamaguchi (1986)
C. domestica	Stem tip (bud of rhizome)	RN + 2% sucrose +0.8% agar	BA (10) + 2, 4-D (1)	Callus	Mukhri and Yamaguchi (1986)
C. domestica	Slice of rhizome	MS	NAA (1) + kinetin (0.1)	Callus	Yasuda *et al.* (1988)
C. longa	Sprouting buds from stored rhizomes	MS + 40gm/lsucrose	Kinetin (0.2) + BAP (0.4) + GA_3 (0.01)	Callus	Shetty *et al.* (1982)
C. longa	Buds	MS	BA (1) + 2,4-D (0.5-1) or BA (3) + 2,4-D (0.1) or BA (5) + 2,4-D (0.1)	Maximum callus	Neera *et al.* (1999)
C. longa	Leaf bases along with the enclosed shoot tips (5mm) from shoot cultures	MS + 0.4% agar	Dicamba, picloram (2) or NAA (5) + BA (0.5)	Callus initiation in the dark for a week	Salvi *et al.* (2001)
C. zedoaria	Slice of rhizome	MS	NAA (1) + kinetin (0.1)	Callus	Yasuda *et al.* (1988)
C. aromatica	Slice of rhizome	MS	NAA (1) + kinetin (0.1)	Callus	Yasuda *et al.* (1988)
C. xanthorhiza.	Emerging buds (2-3mm) from rhizomes	RN + 2% sucrose +0.8% agar	BA (10) + NAA (15)	Callus	Mukhri and Yamaguchi (1986)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 7 Summary of *in vitro* shoot induction and regeneration studies on cardamom and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
E. cardamomum	Callus from sprouted seeds	MS + CW (18%)	IAA (1) + BAP (2)	80% shoot bud initiation	Srinivasa Rao *et al.* (1982)
E. cardamomum	Callus from sprouted seeds	MS + CW (10%)	IAA (1) + BAP (2 - 5)	4-6 shoots per subculture	Srinivasa Rao *et al.* (1982)
E. cardamomum	Shoot bud	MS + CW (5%) + Vitamin	BAP (0.5) + kinetin (0.5) + IAA (2)	Shoots, plants	Nadgauda *et al.* (1983)
E. cardamomum	Immature panicles (inflorescence bud)	MS + calcium pantothenate (0.1 mg l^{-1}) + folic acid (0.1 mg l^{-1}) + CW (10%)	NAA (0.5) + BAP (1.0)	Plantlet	Kumar *et al.* (1985)
E. cardamomum	Shoot bud	SH + CW (15%)	NAA (1) + K (1.5) + BAP (3)	Shoots and plants	Reghunath (1989)
E. cardamomum	Shoot bud	SH + CW (12 – 15%)	NAA (0.5 – 1) + K (0.5 - 3) or BA (3 –4)	Multiplication	Reghunath and Bajaj (1992)
E. cardamomum	Shoot meristem (3-4mm) from *in-vitro* shoot proliferation cultures	Pre-cultured in MS basal medium + 1% AC for 7 days	ND	Shoot meristems	Bajaj *et al.* (1993)
E. cardamomum	Shoot meristems	MS + CW (5%) + agar (5 gm/l)	BA (2 – 3) + kinetin (0.5 – 1)	Shoot regeneration	Bajaj *et al.* (1993)
E. cardamomum	Young vegetative buds	MS + CW (20%) + agar (6 gm/l)	NAA (0.5) + IBA (0.2) + BA (1.0) + Kinetin (0.2)	Shoot multiplication	Lukose *et al.* (1993)
A. subulatum	Young sprouting buds from rhizome	MS + sucrose (3%)	Kinetin (0.5)	Shoot	Sajna *et al.* (1997)
A. subulatum	Shoot	MS medium	BA (1) + IBA (0.5)	Shoot multiplication	Sajna *et al.* (1997)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 8 Summary of *in vitro* shoot induction and regeneration studies on ginger and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
Z. officinale	Buds (2 – 3mm) from rhizomes in storage	MS macro elements + RN vitamins and minor elements + sucrose (2%) + agar (0.8%)	BA (1)	Plantlet	Hosaki and Sagawa (1977)
Z. officinale	Emerging buds of shoot tips (c. 2mm)	MS macro elements + RN vitamins and trace elements + sucrose (4%) + agar (0.8%)	BA (1)	Plantlet	De Lange *et al.* (1987)
Z. officinale	Pale yellow sprouting buds	MS + agar (0.8%)	Hormone free media	Growth of buds in to 10-25 mm shoots	Bhagylakshmi and Singh (1988)
Z. officinale	Meristems with only apical dome or one or two leaf primordial (0.1- 0.5mm)	MS ($3/4^{th}$) + sucrose (6%) + CW (20%) + AA (100 mg l^{-1}) + GL (400 mg l^{-1}) + AC (250 mg l^{-1}) + agar (0.8%)	IBA (0.5) + IBA (0.4)	Shoots	Bhagylakshmi and Singh (1988)
Z. officinale	Meristems with only apical dome or one or two leaf primordial (0.1- 0.5mm)	MS ($3/4^{th}$) + sucrose (3%) + AA (100 mg l^{-1}) + AC (100 mg l^{-1}) + agar (0.8%)	BAP (4 – 5)	Shoot multiplication	Bhagylakshmi and Singh (1988)
Z. officinale	Shoot tips (2 – 3mm)	Modified MS + 20gm sucrose	BA (5) + NAA (0.5)	Shoot initiation and multiplication	Inden *et al.* (1988)
Z. officinale	Leafy aerial pseudostem	MS + *myo*-Inositol (100) + sucrose (3%) + agar (0.3%)	BA (11μM) + NAA (0.6μM)	Plantlet	Ikeda and Tanabe (1989)
Z. officinale	Decapitated crown sections	Liquid media with MS salts + *myo*-Inositol (100) + sucrose (3%)	BA (11μM)	Plantlet	Ikeda and Tanabe (1989)
Z. officinale	Stem tip (rhizome buds)	MS + sucrose (3%) + agar (0.8%)	BAP (3)	Plantlet	Keshavachandran and Khader (1989)
Z. officinale	Callus	Revised MS (MS major salts + RN minor elements + organic addenda + sucrose (2%) + agar (0.8%)	BA (1 – 3)	Shoot	Malamug *et al.* (1991)
Z. officinale	ND	Revised MS (MS major salts + RN minor elements + organic addenda + sucrose (2%) + agar (0.8%)	NAA (5) + BA (1), and NAA (1) + BA (5)	Plantlet	Malamug *et al.* (1991)

Appendix Table 8 (cont'd)

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
Z. officinale	Axillary bud from rhizome	MS	BA (4.4μM)	Plantlet	Dekkers *et al.* (1991)
Z. officinale	ND	MS	2,4-D (0.9μM) + BA (44.4μM) or kinetin (46.5μM)	Organogenesis and plantlet formation	Nirmal Babu *et al.* (1992)
Z. officinale	Inflorescence axis sections with bract base and vegetative axillary bud	Liquid medium of ½-MS salts and organics + 15% CW + 2% sucrose (where media changed with fresh ones at monthly interval)	ND	Growth of buds	Brain and Richard (1993)
Z. officinale	Inflorescence axis sections with bract base and vegetative axillary bud	Agar solidified ½-MS basal salts and organics +2% sucrose	4.4μM BAP	Shoot induction	Brain and Richard (1993)
Z. officinale	Embryogenic callus	MS	BA (8.9μM)	Shoot regeneration	Kacker *et al.* (1993)
Z. officinale	Shoots from tissue culture	MS	BAP (1) + calcium pantothenate (2) +GA_3 (0.2)+ NAA (0.05)	Shoot proliferation	Sharma and Singh (1995)
Z. officinale	Shoots from tissue culture	MS + 75gm/l sucrose	BAP (8)	Microrhizome induction	Sharma and Singh (1995)
Z. officinale	Small actively growing buds (ca 0.5cm)	MS + 3% sucrose + 8% agar	Kinetin (2)	Shoot multiplication	Sharma and Singh (1997)
Z. officinale	Rhizome buds	MS	BAP (3)	Axillary bud proliferation	Tyagi *et al.* (1998)
Z. officinale	Bud explants	MS	BAP (0.1) + K (0.1)	Plantlet	Devi (1999)
Z. officinale	Plantlet	MS	BAP (4)	Shoot production	Devi (1999)
Costus spp	Aerial stem nodes	MS + AC (0.3gm/l)	BA (4.4μM)	Plantlet	Dekkers *et al.* (1991)
K. galanga	Callus	MS	BAP (0.5) + NAA (0.05)	Small green shoot buds	Vincent *et al.* (1991)
K. galanga	Callus	MS	NAA (1) + BAP (1.5)	Bud multiplication	Vincent *et al.* (1991)
A. purpurata	Bulbils arising in the inflorescence	MS + AC (0.3gm/l)	BA (4.4μM)	Plantlet	Dekkers *et al.* (1991)
Z. cassumunar	Excised shoot tips	LS + sucrose (20 g l^{-1}) + agar (10 g l^{-1})	BAP (1 or 2)	Buds	Poungpet and Krisana (1993)

Appendix Table 8 (cont'd)

Species	Explants	Medium [1/]	PGR [1/]	Growth response	References
A. purpurata.	Inflorescence bud	MS salts (1/2- strength macronutrients) + thiamin (2 mg l^{-1}) + nicotinic acid (1 mg l^{-1}) + pyridoxine (2 mg l^{-1}) + *myo*-Inositol (100 mg l^{-1}) + CH (500 mg l^{-1}) + sucrose (2%) + gerlite (2gm/l)	IAA (5µM) or NAA (5µM) + BA (10µM)	Bud establishment	Illg and Faria (1995)
A. purpurata	ND	MS salts (1/2- strength macronutrients) + thiamin (2 mg l^{-1}) + nicotinic acid (1 mg l^{-1}) + pyridoxine (2 mg l^{-1}) +*myo*-Inositol (100 mg l^{-1}) + CH (500 mg l^{-1}) + sucrose (2%) + gerlite (2gm/l)	BA (10µM) + NAA (5µM)	Multiple shoot	Illg and Faria (1995)
A. purpurata	Inflorescence bud	MS salts (1/2 strength) + thiamin (2 mg l^{-1}) + nicotinic acid (1 mg l^{-1}) + pyridoxine (2 mg l^{-1}) + *myo*-Inositol (100 mg l^{-1}) + CH (500 mg l^{-1}) + sucrose (20gm/l) + gerlite (2gm/l)	IAA (5µM) or NAA (5µM) + BA (10µM)	Bud establishment	Illg and Faria (1995)
Z. spectabile	Axillary buds	MS salts (1/2 strength) + thiamin (2 mg l^{-1}) + nicotinic acid (1 mg l^{-1}) + pyridoxine (2 mg l^{-1}) + *myo*-Inositol (100 mg l^{-1}) + CH (500 mg l^{-1}) + sucrose (20gm/l) + gerlite (2gm/l)	IAA (5µM) or NAA (5µM) + BA (10µM)	Bud establishment	Faria and Illg (1995)
Z. spectabile	Axillary buds	MS/2	BA (10µM)	Shoot elongation and proliferation	Faria and Illg (1995)
A. galanga	Emerging buds of rhizomes	MS + sucrose (3%) + agar (0.8%)	Kinetin (3.0)	Shoot	Borthakur *et al.* (1999)
K. galanga	Rhizome pieces	MS	BA (12 µM)	Rooting	Shirin *et al.* (2000)
Z. purpureus	Rhizome buds	MS	BAP (3)	Axillary bud proliferation	Tyagi *et al.* (1998)
Z. weightanium	Rhizome buds	MS	BAP (3)	Axillary bud proliferation	Tyagi *et al.* (1998)
Z. zerumbet	Rhizome buds	MS	BAP (3)	Axillary bud proliferation	Tyagi *et al.* (1998)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 9 Summary of *in vitro* shoot induction and regeneration studies on turmeric and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
C. domestica	Stem tip	MS + CW (10%)	Kinetin (0.1%) + BA (0.2%)	Plantlet	Nadgauda *et al.* (1978)
C. domestica	Buds excised from sprouting rhizomes	MS	BAP (2) + kinetin (1)	Shoot induction	Kuruvinashetti and Iyer (1982)
C. longa	Sprouting buds from stored rhizomes	MS + 40gm/lsucrose	Kinetin (0.2) + BAP (0.4) + GA_3 (0.01)	Shoot regeneration	Shetty *et al.* (1982)
C. domestica	Callus	RN + 2% sucrose +0.8% agar	BA (1) + NAA (1)	Shoots	Mukhri and Yamaguchi (1986)
C. domestica	Stem tip (bud of rhizome)	RN + 2% sucrose +0.8% agar	BA (1)	Shoot	Mukhri and Yamaguchi (1986)
C. domestica	Callus	RN + 2% sucrose +0.8% agar	BA (10)	Shoot	Mukhri and Yamaguchi (1986)
C. longa	Stem tip	MS	Kinetin (1)+ Phloroglusinol (1mM)	Shoot, plantlet	Sato *et al.* (1987)
C. longa	Shoot explant	MS	BA (10)	Shoot formation	Sato *et al.* (1987)
C. domestica	Young sprouting buds from rhizome	MS + 40 g l^{-1} sucrose + 7gm/l agar	BAP (1)	Shoot growth	Keshavachandran and Khader (1989)
C. longa	Bud tissue	MS + sucrose (40gm/l)	Kinetin (1) + BA (1)	Plantlet	Keshavachandran and Khader (1989)
C. domestica	Stem tip (bud of rhizome)	MS + sucrose (30 g l^{-1}) + agar (0.8%)	BAP (3.0)	Plantlet	Balachandran *et al.* (1990)
C. longa	Rhizome buds	MS	BAP (3)	Axillary bud proliferation	Tyagi *et al.* (1998)

Appendix Table 9 (cont'd)

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
C. longa	Callus from leaf bases	MS + 0.25% phytagel	BA (5) + TIBA (0.1) or 2,4-D (0.1)	Callus regeneration (formation of green shoot primordia)	Salvi *et al.* (2001)
C. longa	Regenerated shoots from callus	½ MS + 2% sucrose	Kinetin (1)	Complete shoot	Salvi *et al.* (2001)
C. longa	Buds from field grown rhizomes	MS + B5 vitamins + 0.8% agar + 30 gm sucrose	BA (10µM) + NAA (1µM)	Initial shoot multiplication	Salvi *et al.* (2002)
C. longa	Buds from field grown rhizomes	MS + 0.4% agar	BA (5µM) + NAA (1µM)	Multiplication and maintenance of shoots	Salvi *et al.* (2002)
C. longa	Actively growing shoots	MS + 0.55% Agargel + 3% sucrose	BA (13.32 µM)	Culture initiation	Prathanturarug *et al.* (2003)
C. longa	*In vitro* grown shoot explants	MS + 0.55% Agargel + 3% sucrose	TDZ (18.17 µM)	Shoot multiplication	Prathanturarug *et al.* (2003)
C. aeruginosa	Rhizome buds	MS	BAP (3)	Axillary bud proliferation	Tyagi *et al.* (1998)
C. aeruginosa	Stem tip (bud of rhizome)	MS + sucrose (30 g l^{-1}) + agar (0.8%)	BAP (3.0)	Plantlet	Balachandran *et al.* (1990)
C. aeruginosa	Axillary bud from rhizome	MS	BA (4.4µM)	Plantlet	Dekkers *et al.* (1991)
C. alismatifolia	Young inflorescence (at the stage where all their bracts were tightly close), and Axillary buds from field grown rhizomes	MS + Sucrose (3%)	BA (13.32 mmol/l)	Shoot multiplication	Wannakrairoj (1997)
C. amada	Axillary bud from rhizome	MS	BA (4.4µM)	Plantlet	Dekkers *et al.* (1991)
C. caesia	Stem tip (bud of rhizome)	MS + sucrose (30 g l^{-1}) + agar (0.8%)	BAP (3.0)	Plantlet	Balachandran *et al.* (1990)
C. caesia	Rhizome buds	MS	BAP (3)	Axillary bud proliferation	Tyagi *et al.* (1998)
C. xanthorhiza	Callus	RN + 2% sucrose +0.8% agar	BA (1) + NAA (1)	Shoots	Mukhri and Yamaguchi (1986)
C. zedoaria	Stem tip	MS	NAA (0 – 1) or BA (0 - 3)	Plantlet	Yasuda *et al.* (1988)
C. aromatica	Rhizome sprouts	MS	BA (5)	Shoot multiplication	Nayak (2000)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 10 Summary of *in vitro* root induction studies on cardamom and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
E. cardamomum		½ MS + agar + AC (0.5 - 1)	Then transfer it to MS ½ -liquid state with IBA (1 – 1.5%)	Rooting	Reghunath and Bajaj (1992)
E. cardamomum	Shoot meristems	½ MS + agar + AC (0.5 1%)	ND	Rooting	Bajaj *et al.* (1993)
E. cardamomum	Shoots	White's medium	NAA (0.5)	Rooting	Lukose *et al.* (1993)
A. subulatum	ND	MS medium	BA (1) + IBA (0.5)	Rooting	Sajjna *et al.* (1997)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 11 Summary of *in vitro* root induction studies on ginger and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
Z. officinale	Shoot tips (2 – 3mm)	Modified MS + 20gm sucrose	BA (5) + NAA (0.5)	Root formation	Inden *et al.* (1988)
Z. officinale	ND	MS in liquid media	NAA (5.4μM)	Rooting	Nirmal Babu *et al.* (1992)
Z. officinale	Shoots	Agar solidified ½-MS basal salts and organics +2% sucrose	No growth regulators	Rooting	Brain and Richard (1993)
Z. officinale	ND	MS	NAA (16.1μM) + 2,4-D (13.6 – 22.6μM) + Dicamba (5.4 – 9.0μM)	Rooting	Kacker *et al.* (1993)
Z. officinale	ND	LS + sucrose (20 g l^{-1}) + agar (10 g l^{-1}) + AC (20 g l^{-1})	BAP (1)	Root	Poungpet and Krisana (1993)
Z. officinale	ND	MS	Growth regulator free	Root	Devi (1999)
A. purpurata	ND	Sterile water or in liquid or gerlite-gelled medium	With or without IAA or NAA (5μM)	Rooting	Illg and Faria (1995)
A. purpurata	ND	MS salts (1/2 strength) + thiamin (2 mg l^{-1}) + nicotinic acid (1 mg l^{-1}) + pyridoxine (2 mg l^{-1}) + *myo*-Inositol (100 mg l^{-1}) + CH (500 mg l^{-1}) + sucrose (20gm/l) + gerlite (2gm/l)	IAA (10μM) or NAA (10μM)	Rooting	Illg and Faria (1995)
A. galanga	Emerging buds of rhizomes	MS + sucrose (3%) + agar (0.8%)	Kinetin (3.0)	Rooting	Borthakur *et al.* (1999)
A. galanga		MS with 10 mg l^{-1} thiamin (i.e. to replace the standard vitamins in MS) + 3% sucrose + 0.8% agar	kinetin (3.0)	Root growth and elongation	Borthakur *et al.* (1999)
K. galanga	Shoots	½-MS	BA (12 μM)	Rooting	Shirin *et al.* (2000)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 12 Summary of *in vitro* root induction studies on turmeric and its close relatives.

Species	Explants	Medium [1/]	PGR (mg l^{-1}) [1/]	Growth response	References
C. domestica	ND	W + Na_2MoO_4(0.25) + $CuCl_2$(0.25)	ND	Root	Nadgauda *et al.* (1978)
C. domestica	ND	½-MS liquid minerals + 20gm/l sucrose	ND	Root induction	Kuruvinashetti and Iyer (1982)
C. domestica	Callus	RN + 2% sucrose +0.8% agar	BA (1) + NAA (1)	Roots	Mukhri and Yamaguchi (1986)
C. domestica	Emerging buds (2-3mm) from rhizomes	RN + 2% sucrose +0.8% agar	BA (10) + NAA (15)	Embryoids and roots	Mukhri and Yamaguchi (1986)
C. domestica	Stem tip (bud of rhizome)	RN + 2% sucrose +0.8% agar	BA (1)	Roots	Mukhri and Yamaguchi (1986)
C. domestica	Young sprouting buds excised from rhizome	MS + 40 g l^{-1} sucrose + 7gm/l agar	BAP (1)	Rooting	Keshavachandran and Khader (1989)
C. longa	Shoots	½-MS	0.1 mg l^{-1} NAA	Rooting	Sato *et al.* (1987)
C. longa	Regenerated shoots	½ MS	PGR-free	Complete rooted plantlets	Salvi *et al.* (2001)
C. xanthorhiza	Callus	RN + 2% sucrose +0.8% agar	BA (1) + NAA (1)	Roots	Mukhri and Yamaguchi (1986)
C. xanthorhiza	Stem tip (bud of rhizome)	RN + 2% sucrose +0.8% agar	BA (10)	Root and bud	Mukhri and Yamaguchi (1986)
C. xanthorhiza	Callus	RN + 2% sucrose +0.8% agar	ND	Root	Mukhri and Yamaguchi (1986)
C. aromatica	Rhizome sprouts	MS	BA (5)	Rooting	Nayak (2000)

[1/] MS = Murashige and Skoog (1962); RN= Ringe and Nitsch (1968); W= White (1963); LS= Linsmaier and Skoog (1965); B5= Gamborg *et al.* (1968); BAP or BA= 6-benzylaminopurine or N^6-benzyladenin; NAA= α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA= Indol-3-acetic acid; IBA= Indol-3-butyric acid; K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid; GL= Gerlite; AA= Ascorbic acid; AC= Activated charcoal; CH= casein hydrolsate; ND= not described in the report.

Appendix Table 13 Measurements of stomata from the abaxial side of korarima leaves (from preliminarily selected plants based on morphological features).

Source	Plant No.	Stomata Number		Stomata Length		Stomata Index	
		Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
Control	**1**	9.00c ±1.05	11.71	11.50jk ± 1.08	9.39	8.81b ± 0.82	9.29
	2	8.80c ± 0.79	8.96	11.60j ± 1.17	10.12	8.90b ± 0.69	7.70
	3	9.90b ± 1.10	11.12	11.60j ± 1.26	10.90	9.88b ± 0.87	8.83
	4	10.70a ± 1.06	9.90	11.40jk ± 1.17	10.30	9.93b ± 1.16	11.66
	5	9.50bc ± 0.53	5.55	10.80k ± 0.92	8.51	13.48a ± 1.52	11.27
Colchicine (125 μM l^{-1})	**6**	3.80e-h ± 0.63	16.64	14.50ih ± 0.71	4.88	6.02d-h ± 1.20	19.97
	7	3.30gh ± 0.48	14.64	15.50c-g ± 0.71	4.56	5.20gh ± 0.68	13.05
	8	3.00h ± 0.82	27.22	15.10d-h ± 0.57	3.76	5.31fgh ± 1.56	29.37
	9	2.20i ± 0.42	19.17	20.60a ± 1.07	5.22	5.19gh ± 1.29	24.96
	10	3.50fgh ± 0.71	20.20	16.90b ± 0.57	3.36	5.29fgh ± 0.93	17.55
Colchicine (250 μM)	**11**	3.40fgh ± 0.84	24.80	15.10d-h ± 0.74	4.89	4.75h ± 1.30	27.49
	12	3.40fgh ± 0.70	20.56	14.50ih ± 0.53	3.63	5.38fgh ± 1.05	19.54
Colchicine (375 μM l^{-1})	**13**	3.50fgh ± 0.53	15.06	14.40ih ± 0.52	3.59	5.90d-h ± 0.90	15.20
	14	3.60fgh ± 0.52	14.34	15.10d-h ± 0.88	5.80	6.21c-g ± 0.81	12.99
Colchicine (500 μM l^{-1})	**15**	3.90efg ± 0.99	25.50	14.80f-I ± 0.42	2.85	6.21c-g ± 1.81	29.20
	16	3.60fgh ± 0.97	26.84	15.80cde ± 0.79	4.99	6.75cde ± 1.27	18.83

Appendix Table 13 (cont'd)

Source	Plant No.	Stomata Number		Stomata Length		Stomata Index	
		Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
Oryzalin (10 μM l^{-1})	**17**	3.40fgh ± 0.70	20.56	15.00e-i ±0.94	6.29	5.76e-h ±1.54	26.75
	18	3.30gh ±0.67	20.45	15.50c-g ±0.53	3.40	6.93ecd ±1.17	16.88
	19	3.90efg ± 0.57	14.56	15.40c-g ±0.52	3.35	6.61c-f ±1.04	15.69
	20	3.70fgh ±0.82	22.25	15.60c-f ±0.70	4.48	6.21c-g ±1.67	26.85
	21	3.60fgh ±0.70	19.42	16.00c ±0.94	5.89	6.81cde ±1.48	21.71
	22	3.90efg ± 0.74	18.92	15.60c-f ±0.52	3.31	7.14cd ±1.40	19.56
	23	3.40fgh ± 0.52	15.19	15.60c-f ±0.52	3.31	5.93d-h ±1.03	17.36
	24	3.90efg ±0.74	18.92	15.00e-i ±0.67	4.44	5.25fgh ±0.95	18.08
	25	4.10efg ±0.74	18.00	15.20c-h ±0.63	4.16	7.39c ±1.61	21.81
Oryzalin (20 μM l^{-1})	**26**	3.50fgh ± 0.53	15.06	15.70cde ±0.95	6.04	5.86d-h ±0.99	16.85
	27	3.90efg ±0.74	18.92	15.90cd ±1.20	7.53	6.86cde ±1.61	23.40
	28	4.60ed ±0.84	18.33	15.00e-i ±0.82	5.44	5.33fgh ±0.85	15.98
	29	3.60fgh ±0.97	26.84	15.70cde ±0.67	4.30	6.28c-g ±1.68	26.78
	30	5.10d ±1.10	21.58	14.20i ± 0.42	2.97	6.78cde ±1.46	21.54
	31	4.20ef ± 0.92	21.88	14.70ghi 0.48	3.29	7.01cde ±1.67	23.78
Oryzalin (60 μM l^{-1})	**32**	4.20ef ±0.79	18.78	15.60c-f ± 0.70	4.48	6.40c-g ±1.26	19.73
	Model						
	Prob.	***		***		***	
	% CV	16.93		5.38		18.81	

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Appendix Table 14 Summary of simple statistics of stomata from the abaxial side of korarima leaves by treatment group (excluding non-respondent treatments).

Source	Stomata Number		Stomata Length		Stomata Index	
	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
Control	9.58a ± 1.13	11.76	11.38d ± 1.120	9.87	10.20a ± 2.00	19.58
125 μM l^{-1} colch.	3.16d ± 0.82	25.86	16.52a ± 2.32	14.06	5.40cd ± 1.17	21.60
250 μM l^{-1} colch.	3.40cd ± 0.75	22.17	14.80c ± 0.70	4.70	5.06d ± 1.20	23.66
375 μM l^{-1} colch.	3.55cd ± 0.51	14.38	14.75c ± 0.79	5.33	6.06c ± 0.85	13.96
500 μM l^{-1} colch.	3.75bc ± 0.97	25.77	15.30bc ± 0.80	5.24	6.48b ± 1.55	23.91
10 μM l^{-1} Oryz.	3.69bc ± 0.71	19.34	15.43bc ± 0.72	4.66	6.45b ± 1.45	22.45
20 μM l^{-1} Oryz.	4.15b ± 1.01	24.23	15.20bc ± 0.99	6.50	6.35b ± 1.48	23.34
40 μM l^{-1} Oryz.	NP	NP	NP	NP	NP	NP
60 μM l^{-1} Oryz.	4.20b ± 0.79	18.78	15.60b ± 0.70	4.48	6.40b ± 1.26	19.73
80 μM l^{-1} Oryz.	NP	NP	NP	NP	NP	NP
Model Prob.	***		***		***	
% CV	19.00		8.25		21.88	

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.
NP = no putative shoots.

Appendix Table 15 Measurements of stomata from the abaxial side of krawan leaves (from preliminarily selected plants based on morphological features).

Source	Plant No.	Stomata number		Stomata length		Stomata index	
		Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
Control	**1**	15.70cd ± 1.34	8.52	12.20j ± 0.79	6.47	11.91d-g ± 1.66	13.94
	2	16.10cd ± 1.60	9.91	11.40j ± 0.97	8.47	13.76a-d ± 2.08	15.11
	3	17.90b ± 2.23	12.48	11.90j ± 0.99	8.36	10.62f-j ± 1.31	12.31
	4	14.90de ± 0.74	4.95	12.00j ± 1.05	8.78	14.07abc ± 1.71	12.16
Colchicine (125 μM l^{-1})	**5**	10.80g-j ± 1.75	16.21	14.40c-g ± 0.52	3.59	9.09jk ± 1.82	20.00
	6	10.50g-k ± 1.51	14.37	14.40c-g ± 0.70	4.86	10.05g-j ± 1.72	17.12
	7	10.60g-k ± 1.26	11.93	14.60b-g ± 0.70	4.79	7.81k ± 1.19	15.26
	8	12.10fg ± 1.20	9.89	14.30d-g ± 0.48	3.38	10.21g-j ± 0.84	8.23
Oryzalin (10 μM l^{-1})	**9**	10.90g-j ± 2.13	19.56	14.60b-g ± 0.70	4.79	9.91hij ± 1.43	14.41
	10	16.70bc ± 2.21	13.26	14.70b-f ± 0.82	5.60	13.13a-d ± 2.24	17.09
	11	11.00g-j ± 1.05	9.58	14.60b-g ± 0.70	4.79	12.64a-e ± 1.56	12.35
	12	10.50g-k ± 1.43	13.65	15.20abc ± 0.92	6.05	13.08a-d ± 1.49	11.42
	13	13.60ef ± 1.17	8.63	14.50c-g ± 0.71	4.88	12.15c-f ± 1.23	10.14

Appendix Table 15 (cont'd)

Source	Plant No.	Stomata number		Stomata length		Stomata index	
		Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
Oryzalin (20 μM l^{-1})	**14**	11.30g-j ± 1.25	11.08	14.40c-g ± 0.52	3.59	11.05e-i ± 1.63	14.77
	15	10.10h-k ± 1.37	13.57	15.20abc ± 0.42	2.77	11.16e-h ± 1.39	12.45
	16	13.70ef ± 1.64	11.94	13.20hi ± 0.42	3.19	12.75a-e ± 1.44	11.33
	17	11.50ghi ± 1.08	9.39	16.00a ± 1.25	7.80	14.48a ± 1.24	8.59
	18	11.50ghi ± 1.51	13.12	14.60b-g ± 0.52	3.54	12.84a-e ± 1.94	15.08
	19	10.60g-k ± 1.65	15.53	14.70b-f ± 0.48	3.29	11.10e-h ± 2.32	20.88
	20	9.60jk ± 2.22	23.14	14.20d-g ± 0.42	2.97	9.27ijk ± 1.91	20.62
	21	14.50de ± 1.58	10.90	15.40ab ± 0.97	6.27	11.88d-g ± 1.75	14.73
	22	16.20cd ± 2.94	18.13	15.90a ± 0.88	5.51	14.16ab ± 2.50	17.66
Oryzalin (40 μM l^{-1})	**23**	21.70a ± 3.33	15.37	11.90j ± 1.20	10.06	13.50a-d ± 2.49	18.42
	24	18.00b ± 2.40	13.35	13.10i ± 1.85	14.14	13.23a-d ± 2.18	16.51
	25	10.90g-j ± 1.29	11.80	14.50c-g ± 0.53	3.63	12.07def ± 1.97	16.32
	26	8.90k ± 1.10	12.37	15.00bcd ± 0.94	6.29	10.97e-i ± 1.07	9.78
	27	11.30g-j ± 0.82	7.29	14.50c-g ± 0.53	3.63	14.51a ± 1.62	11.17
	28	15.90cd ± 1.45	9.11	14.10efg ± 0.99	7.05	13.22a-d ± 1.44	10.86
	29	9.60jk ± 1.17	12.23	14.80b-e ± 0.63	4.27	12.69a-e ± 2.07	16.29
	30	9.70ijk ± 1.83	18.85	15.90a ± 0.74	4.64	13.17a-d ± 2.12	16.11
Oryzalin (80 μM l^{-1})	**31**	11.40g-j ± 2.22	19.48	13.90fgh ± 0.74	5.31	12.53b-e ± 2.46	19.64
	32	11.70gh ± 1.64	13.99	13.80ghi ± 0.42	3.06	12.35b-f ± 1.69	13.71
Model							
Prob.		***		***		***	
% CV		13.50		5.80		14.83	

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Appendix Table 16 Summary of simple statistics of stomata from the abaxial side of krawan leaves by treatment group (excluding non-respondent treatments).

Source	Stomata number		Stomata length		Stomata index	
	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
Control	16.15a ± 1.87	11.61	11.88d ± 0.97	8.13	12.59a ± 2.18	17.28
125 μM l^{-1} colch.	11.00b ± 1.54	13.96	14.43b ± 0.59	4.12	9.29b ± 1.69	18.24
250 μM l^{-1} colch.	NP	NP	NP	NP	NP	NP
375 μM l^{-1} colch.	NP	NP	NP	NP	NP	NP
500 μM l^{-1} colch.	NP	NP	NP	NP	NP	NP
10 μM l^{-1} Oryz.	10.80bc ± 1.56	14.47	14.80ab ± 0.81	5.44	11.88a ± 2.03	17.10
20 μM l^{-1} Oryz.	10.77bc ± 1.66	15.43	14.85a ± 0.88	5.92	11.65a ± 2.37	20.30
40 μM l^{-1} Oryz.	10.08c ± 1.52	15.11	14.94a ± 0.84	5.64	12.68a ± 2.10	16.58
60 μM l^{-1} Oryz.	NP	NP	NP	NP	NP	NP
80 μM l^{-1} Oryz.	11.55b ± 1.90	16.49	13.85c ± 0.59	4.24	12.44a ± 2.06	16.55
Model Prob.	***		***		***	
% CV	14.28		5.74		18.01	

Treatment means within a column followed by the same letter are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level of probability.
NP = no putative shoots.