Journal of the Rubber Research Institute of Sri Lanka, (1996) 77, 1-14

THE EFFECT OF THIDIAZURON ON AXILLARY SHOOT PROLIFERATION OF *HEVEA BRASILIENSIS IN VITRO*

Priyani Seneviratne and Andrew Flegmann[1]

(Accepted 7 September 1994)

ABSTRACT

Multiple axillary shoot production with a shoot doubling time of 26 days was obtained from nodal explants of juvenile origin *Hevea brasiliensis* on a medium containing thidiazuron (N-phenyl- N'1,2,3-thidiazol-5-ylyrea) at 0.02 ppm in combination with NAA at 0.2 ppm. The clusters of axillary buds produced in the presence of thidiazuron were subcultured at 4-week intervals while subdividing into two to four small propagules depending on the size of the cluster. Axillary buds showed a satisfactory elongation on their transfer onto a growth regulator-free medium. Elongated axillary shoots which were more than 10 mm in height produced roots in the presence of IBA at 2 ppm.

Key words : *Hevea*, micropropagation, rubber, thidiazuron

INTRODUCTION

Hevea brasiliensis is a highly heterozygous open-pollinated perennial tree belonging to the family Euphorbiaceae. It is cultivated mostly in high rainfall tropical areas within 20° north and 20° south of the equator. The importance of natural rubber to the world is vast, with more than 50,000 articles made purely from rubber (Chen, 1984).

The polymerization of isoprene produces polymers having the same overall chemical composition as natural rubber, but the physical properties of this synthetic rubber are inferior in every way. Therefore, for many applications it is necessary to mix the synthetic polymers of isoprene with 30-50% of natural rubber before use.

School of Biology, University of Bath, Claverton Down, Bath BA2 7AY, UK.

Hevea is currently propagated by grafting buds from selected clones onto seedling rootstocks. This has so many disadvantages with intraclonal variation in yield, the most undesirable. It is believed that this variation is caused by the rootstocks that originate from heterozygous seeds (Combe, 1975).

Propagation of *Hevea* by stem cuttings is not recommended as a propagation method because, it is very difficult to induce roots on cuttings and, the root system produced does not provide sufficient anchorage when the tree is fully grown.

Therefore, propagation of *Hevea* via *in vitro* techniques has been attempted since the 1970's. Among the various reports, successful results have been obtained in clonal propagation via shoot-tip and node culture techniques. A monthly multiplication coefficient of 2-3 shoots was obtained by Carron *et al.* (1989) for juvenile-origin nodal explants by incorporating BAP and IBA into culture media. Production of 30 $\pm$ 2 shoots within 165 days has been reported from shoot tip explants of embryo-cultured plants (Goonatilake, 1989) using BAP and IBA.

Thidiazuron is a plant growth regulator that has been used as a cotton defoliant (Arndt *et al.*, 1976; Suttle, 1985). When Mok *et al.* (1982), first reported its cytokinin activity they compared the relative activity of eight cytokinin-active adenine derivatives in promoting the growth of callus tissues of *Phaseolus lunatus.* Thidiazuron and several substituted pyridyl phenylureas have been demonstrated to stimulate *in vitro* meristems and shoot formation at unusually low concentrations (Fellman *et al.*, 1987).

This cytokinin-like activity of thidiazuron has been reported in various tissue culture systems and it has been as effective as. or more than, conventional cytokinins for promotion of shoot proliferation (Prcece *et al.*. 1988; Singha & Bhatiya, 1988) breaking of bud dormancy (Wang *et al.*, 1986) and adventitious shoot regeneration (Cousineu & Donnelly; Elobcidy & Korban, 1988; Fasolo *et al.*. 1989; Imel & Preece, 1988) of a wide range of plant species including several woody species that respond little to conventional cytokinins (Fellman *et al.*, 1987, Kerns & Meyer, 1986, Swartz, 1988; Nieuwkerk *et al.*, 1986).

MATERIALS AND METHODS

Nodal explants, 3-5 cm long, harvested from glass house-grown stock plants were used as explants. Latex was washed off under running tap water and then they were surface sterilized with 70% ethanol for 1-min followed by 10-min immersion in 0.2% $HgCl_2$. A few drops of tween 80 were added to the disinfesting solution prior to treatment. Explants were shaken throughout and then washed 5-6 times in sterile distilled water.

Woody plant medium (Lloyid & McCown, 1980) supplimented with 4% sucrose and 0.6% agar (Lab M-type, MC 2) was used for culture establishment. Growth hormones were introduced into culture media only after the establishment stage of about 4 weeks. All the chemicals used were of analytical grade. Growth regulators and WPM medium were supplied by Sigma Chemicals; thidiazuron was supplied by Schering agriculture, UK.

Media were prepared in 9 cm sterile petri dishes for culture establishment and then in 100 ml steriline jars with screw type lids for the rest of the passages. Rooting of shoots was carried out in 25 mm diameter glass tubes with autoclavable closures.

Media were autoclaved at 121°C and 15 lb/inch^2 pressure for 20 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod. (100 mol $s^{-1}m^{-2}$ by cool white fluorescent bulbs).

The shoot multiplication rate was calculated by using the shoot doubling time formula (Flegmann & Wainwright, 1981).

$N_t = No\, e^{kt}$
where N_t = number of shoots at time t and
N_0 = number of shoot when t=0.

k is the shoot proliferation rate constant. k was calculated from the plot of $\ln N_t/N_0$ against t (time). The proliferation rate constant was converted to shoot doubling time (td) as follows;

$td = \ln 2/k$

The experiment with thidiazuron was carried out in two steps. In the first part, only two distinct levels of thidiazuron (0.1 and 0.002 ppm) were tested. The control medium contained the best combination of kinetin and BAP, 2 ppm and 1 ppm respectively, which had been found to be the best medium for axillary shoot growth in previous experiments. Both thidiazuron containing media contained BAP at 0.2 ppm while all media contained NAA at 0.2 ppm. There were 8 replicates and cultures were recultured every 4 weeks.

In the second part of this experiment, three levels of thidiazuron were tested; control medium contained no growth regulators. The explants used were originated from 6-8 nodal explants that had been multiplied *in vitro* followed by growing them on growth regulator-free medium for 6 weeks. WPM supplied with 4% sucrose and 0.6% agar was used as before. NAA was used at 0.2 ppm and BAP was excluded. Media were prepared in 100 ml steriline jars. There were 10 replicates and cultures were subcultured every 4 weeks.

A peat based compost mixture was used to acclimatize the rooted plantlets in the proragators supplied by the BDH Company, UK.

RESULTS

Results after about 16 weeks showed that the morphology of the axillary shoots produced on thidiazuron containing media was different from that of the cultures grown without thidiazuron (Fig. 1). They were clusters of buds instead of single axillary shoots produced in the kinetin and BAP containing medium.

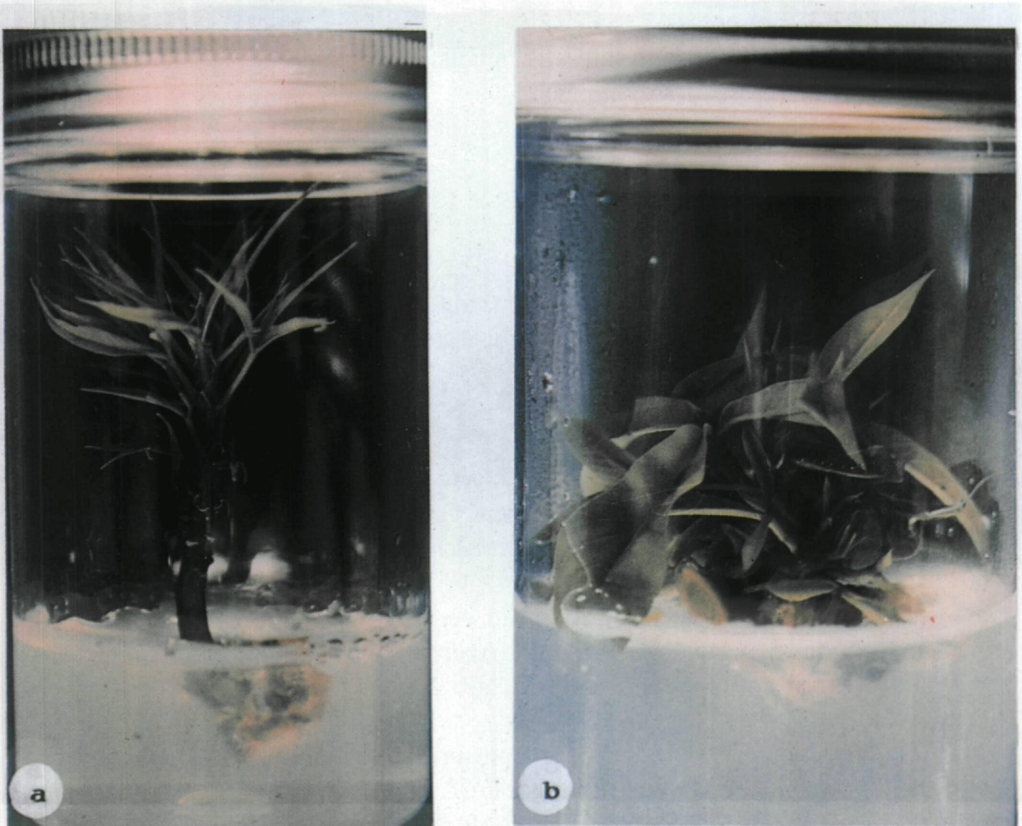

Fig.1 Morphology of axillary shoots produced on (a) control medium (b) thidiazuron containing medium. Results after 16 weeks of culture.

With the control treatment, shoot proliferation occured in steps (Fig. 2a). because there was a period of shoot elongation after each sub culture. The main shortcoming in this pattern of proliferation was that the time required for this period increased gradually. This depressed the rate of shoot proliferation and therefore it was difficult to say whether the proliferation was sustainable or not (Fig. 2a).

In the presence of thidiazuron, the primary nodes produced multiple axillary shoots that continued in the later passages. Therefore, thidiazuron showed a higher proliferation rate compared to conventional cytokinins by producing multiple axillary buds instead of single shoots and seems sustainable (Fig. 2b & c).

The shoot multiplication pattern for the three levels of thidiazuron, ie, 0.002 0.02 and 0.2 ppm for a period of 12 weeks are shown in Fig. 3.

There was no lag phase in this experiment, because all the explants were grown *in vitro* prior to using for this experiment. The growth started soon after their transfer onto particular media (Fig. 3).

After 4 weeks of culture, more than 60% of the cultures on control medium gave only one axillary shoot while the rest of them gave two axillary shoots (Fig. 4a). The shoots produced were 5-20 mm size and contained normal leaves. No multiple axillary shoot formation was observed in any of the cultures on control medium. Roots were formed on about 40% of the cultures at 12 weeks. Shoot doubling time for the cultures grown on control medium was about 33 weeks. Multiple axillary shoot formation with normal leaves was observed in 0.002 ppm thidiazuron medium (Fig. 4b). Axillary shoots produced were 5-15 mm in height. Twenty five percent of the cultures grown on this medium produced roots after 12 weeks. Shoot doubling time was 39 days for this medium. Root formation was not observed in the previous experiment with 0.002 ppm thidiazuron; that medium contained BAP at 0.2 ppm in addition to thidiazuron.

Thidiazuron at 0.02 ppm showed the maximum proliferation; the shoot doubling time was only 23 days. Axillary shoots produced were 5-10 mm in size, and contained a very good leaf growth (Fig. 4c). No root formation was observed in any of the cultures in this medium.

The shoot doubling time for the cultures grown on 0.2 ppm thidiazuron medium was 28 days. But the propagules produced in this medium had no proper axillary shoots as observed on the other two media. They were only clusters of buds that could be divided into small clusters (Fig. 4d). Leaf growth of this medium was poor compared to all other media. No root formation was observed in any of the cultures. Attempts were made to induce the clusters of buds grown on this medium to produce normal axillary shoots by transferring them onto a medium containing only 0.002 ppm thidiazuron. After 4 weeks on lower thidiazuron medium, the propagules kept on producing clusters of buds. But after 8 weeks of culture on the same medium, axillary shoots of 5-10 mm size were produced with normal leaves.

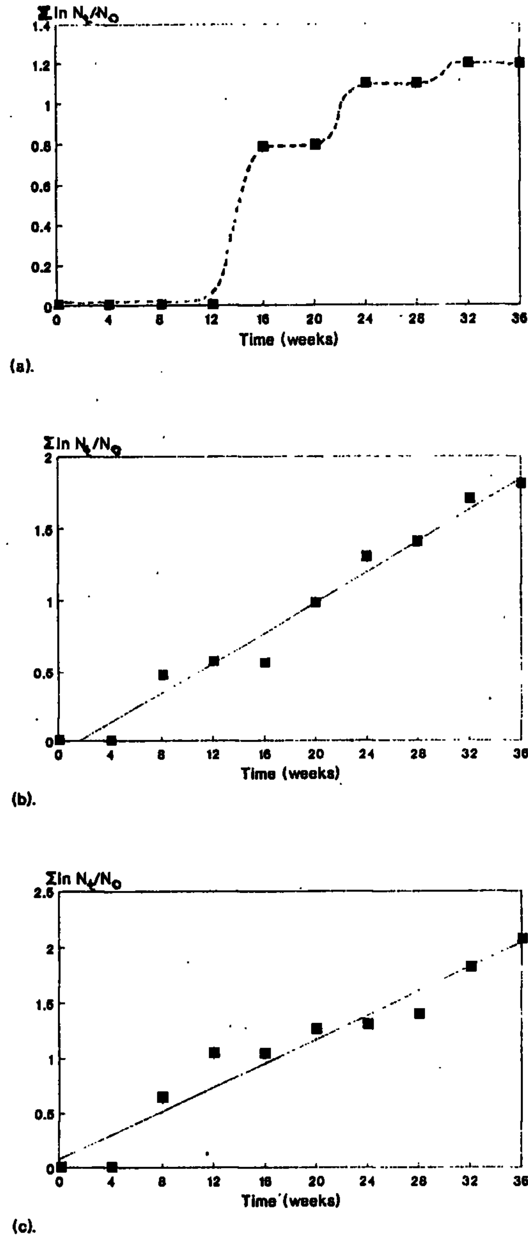

Fig. 2 Shoot doubling time of nodal explants on three media (a) control medium (b) thidiazuron at 0.1 ppm (c) thidiazuron at 0.002 ppm. (n=8)

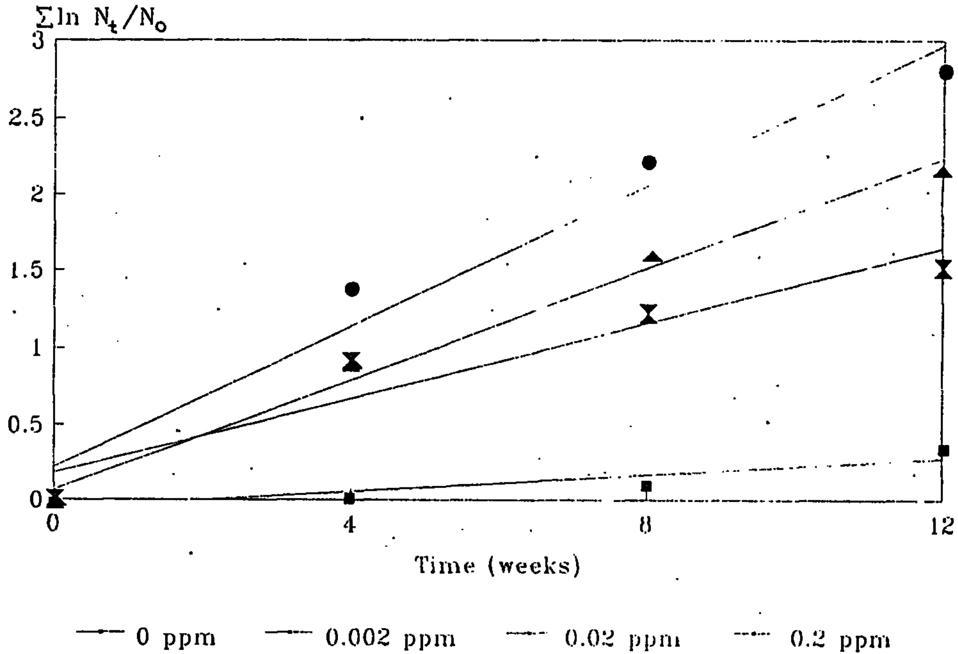

Fig. 3 Shoot doubling time on thidiazuron containing media (n=10).

The single axillary shoots produced on control medium were also tested for their ability to produce multiple axillary shoot in the presence.of thidiazuron. They were transferred onto 0.02 ppm thidiazuron medium, and multiple axillary shoots were observed after 4 weeks of culture.

Rooting of the shoots occured after about 6 weeks after the transfer onto a medium containing 2 ppm IBA. A good leaf growth was observed on elongated apices after about 12 weeks. Rooted plantlets were successfully acclimatized in small propagator trays where high humidity was maintained for the first 2 weeks.

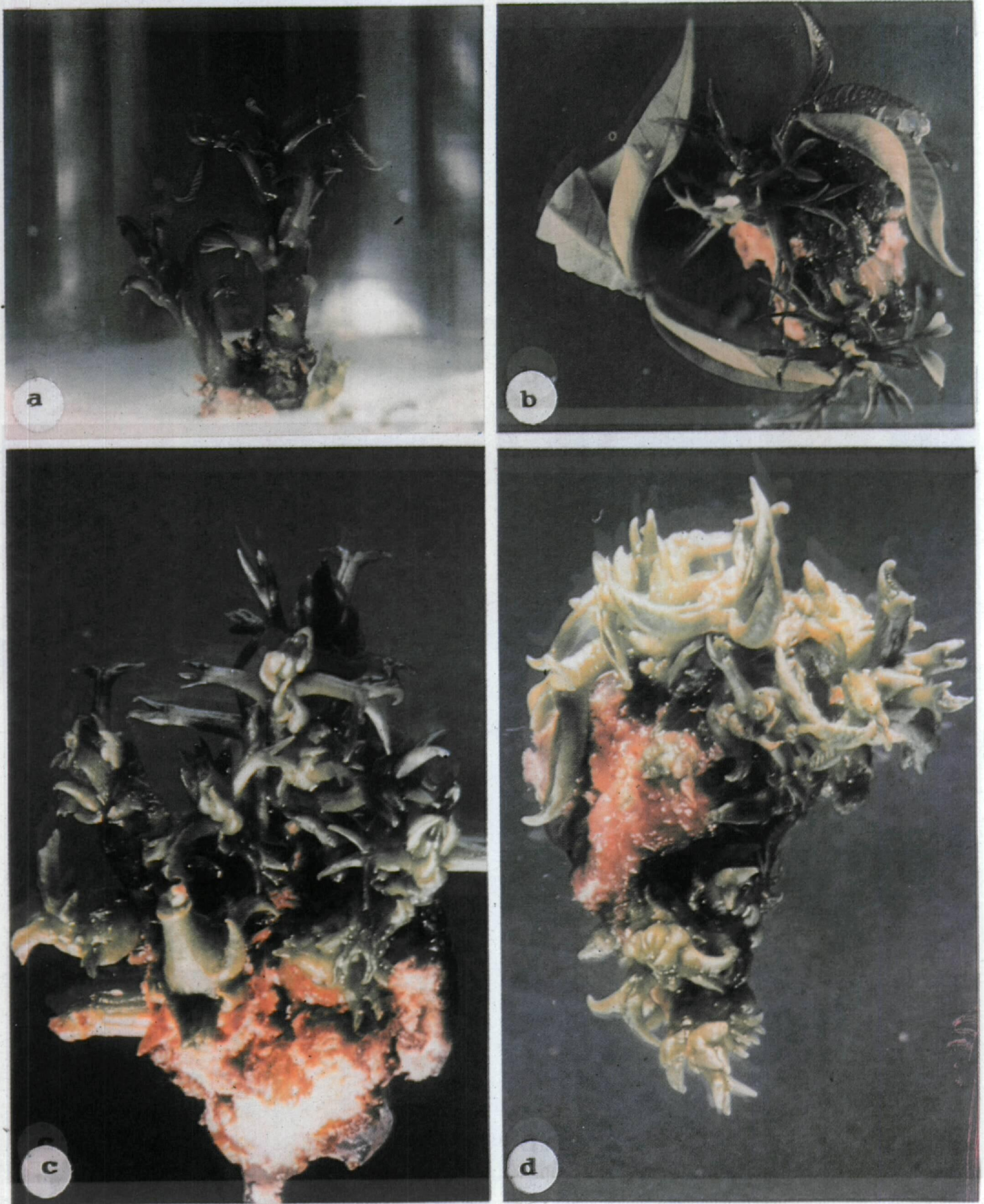

Fig.4 Shoot growth on thidiazuron containing media (a) 0 ppm, (b) 0.002 ppm, (c) 0.02 ppm and (d) 0.2 ppm.

DISCUSSION

The cytokining activity of thidiazuron has been reported with various plant species and in most cases it has been more effective than conventional cytokinings for shoot proliferation and adventitious shoot regeneration.

The strong apical dominance and apparent suppression of axillary meristems that prevented shoot proliferation of *Acer X freemani* was observed by Kerns Meyer (1986) over 4 years while numerous treatments were tested including kinetin, BAP, 2ip, IBA, GA_3 *etc*. The growth of shoot tips continued as single shoots and did not proliferate. But, this situation changed when the shoot tips were transferred to a medium containing 0.0022 - 0.011 ppm thidiazuron and started to proliferate continuously.

In the present studies, BAP at 0.2 ppm was incorporated in one part of the experiment and then thidiazuron was tested with NAA only. Therefore, the effect of BAP was not clear, although the effect of thidiazuron was promising for the axillary shoot proliferation of *Hevea* and obviously encouraging compared to the results obtained with conventional growth regulators.

Concentrations beyond 0.2 ppm were not tested because when comparing 0.1 and 0.002 ppm, the higher concentration showed poor shoot quality and leaf growth. Also, the other workers have used rather low concentrations.

From the range tested, 0.02 ppm showed the maximum proliferation. The shoot doubling times for the media containing 0.002, 0.02 and 0.2 ppm thidiazuron were 39, 23 and 28 days respectively. 45% of the shoots grown on control medium and 25% of them on 0.002 ppm thidiazuron produced roots. Although 0.002 ppm thidiazuron was not strong enough to stop root formation on shoots, it was high enough to induce multiple axillary shoots. Root formation was not observed at this concentration when the BAP at 0.2 ppm was present. Very condensed clusters of buds were produced on 0.2 ppm thidiazuron. But these clusters started to produce normal multiple axillary shoots after 8 weeks of their transfer onto 0.002 ppm thidiazuron medium.

The multiple axillary shoots produced on thidiazuron containing media, developed roots on their transfer onto control medium. The time required for the disappearance of thidiazuron activity on the transfer onto control medium, was dependent on the concentration of thidiazuron in the medium where they had been grown. Also the cultures grown on control medium as single shoots, started to produce multiple axillary shoots on their transfer onto thidiazuron containing medium.

The present findings of the activity of thidiazuron is in agreement with the findings of Nieuwkerk *et al.* (1986). They observed better shoot proliferation of apple in the presence of thidiazuron.

In the present work, when the large clusters of axillary shoots were separated into smaller once, new axillary shoots always emerged from the bases of the clusters, the origin of which were difficult to determine (Fig.5).

As reported by Nieuwkerk *et al.* (1986), after transferring cultures from media containing lower levels of thidiazuron to cytokinin free or growth regulator free medium, proliferation ceased within 3 weeks for most treatments, but continued for up to 15 weeks for explants that had been on 0.22 or 2.2 ppm thidiazuron containing media.

Fig.5 Multiple axillary shoots produced at 0.02 ppm thidiazuron. Results after 20 weeks.

Kerns & Mayer (1985) also reported the effects of thidiazuron on shoot proliferation of red-silver hybrid maples. The prolifics obtained at 0.022 - 0.11 ppm thidiazuron could be separated into 5-7 new propagules at 6 weeks intervals.

Thidiazuron (2.3-4.5 ppm) alone or in combination with BA (1-5 ppm) or kinetin (1-5 ppm) was effective for establishing and for promoting shoot proliferation of grape (*Vitis rotundifolia*). Time of exposure on media containing thidiazuron has been critical for subsequent shoot proliferation. Although cultures initially grew well, tissue browning and senescence were observed on the growing explant during the culture period if the cultures were not regularly transferred (Sudarsono & Goldy, 1991).

Induction of senescence by thidiazuron in cultured lemon (*Citrus limon*) has been reported (Siper & Einset, 1983). They postulated that this was due to thidiazuron stimulating ethylene production, which in turn caused senescence. Transferring cultures every 20 days to fresh medium has overcome browning and senescence of these cultures (Sudarsono & Goldy, 1991).

Thidiazuron was found to be effective on callus inductions and organogenesis as well. Capelle *et al.* (1983) reported that thidiazuron was extremely active in stimulating callus growth of *Phaseolus lunatus*.

Fiola *et al.* (1990) found that thidiazuron was significantly more effective than BAP in inducing organogenesis from detached *Rubus* cotyledons and leaves. The optimum concentration of thidiazuron for organogenesis from cotyledons was 1.1 to 2.2 ppm and for leaves 1.1 to 4.4 ppm. These were much higher than the ranges found optimum for shoot multiplication.

The effect of thidiazuron, with most of the plants, showed that it could be used to proliferate shoot tips or nodes *in vitro*, and that thidazuron was effective at much lower concentrations than conventional cytokinins like BAP. From the cytokinins tested in the present studies thidiazuron was the most effective, where the continuous shoot proliferation was observed.

ACKNOWLEDGEMENTS

The authors wish to express their sincere thanks to the Council of Agricultural Research and Policy of Sri Lanka for the financial support given and to the Rubber Research Institute of Sri Lanka for granting permission for this study and finally to the University of Bath, UK where this study was conducted.

REFERENCES

Arndt, F, Rusch, R, Stillfried, H V, Hanisch, H and Martin, W C (1976). SN 49 537, A new cotton defoliant. *Plant Physiology* Annual meeting supplement **57**, 99.

Capelle, S C, Mok, D W S, Kirchner, S C and Mok, M C (1983). Effects of thidiazuron cytokinin autonomy and the metabolism of N^6-(2-isopentenyl) ($8^{14}C$) adenosine in callus tissues of *Phasiolus lunatus L. Plant Physiology* **73**, 796-802

Carron, M P, Enjalric, F, Lardet, L and Deschamps, A (1989). Rubber (*Hevea brasiliensis* - Muell. Arg.). In: Bajaj, Y P S (Ed) *Biotechnology in Agriculture and Forestry. Vol 5 Trees* (pp 222-245) Springer-Verlag, Berlin.

Chen, Z (1984). Rubber (*Hevea)*. In: Sharp, W R, Evans D A, Ammirato, P V & Yamada, Y (Eds) *Hand book of Plant Cell Culture Vol. 2 Crop Species* (pp 546-571). Macmillan Publishing Co. New York.

Combe, J C (1975). Demonstration of intraclonal variability in young grafted trees. *Revue Generale des Caoutchouces et plastiques* **52**, 91-94

Cousineau, J C and Donnelly, D J (1991). Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. *Plant Cell Tissue and Organ Culture* **27**, 249-255.

Elobeidy, A and Korban, S S (1988). The effect of thidiazuron on shoot regeneration from apple leaf discs. *Hort Science* **23**, 755 (Abstr).

Fasolo F, Zimmerman, R H, and Fordham, I (1989). Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. *Plant Cell Tissue and Organ Culture* **16**, 75-87

Fellman, C D, Read, P E and Hosier, M A (1987). Effects of thidiazuron and CPPU on meristem formation and shoot proliferation. *Hort Science* **22**, 1197-1200

Fiola, J A, Hassan, M A, Swartz, H J, Bors, R H and McNicols, R (1990). Effects of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. *Plant Cell Tissue and Organ Culture* **20**, 223-228.

Flegmann, A W and Wainwright, H (1981). Shoot doubling time: A quantitative parameter for characterizing shoot cultures *in vitro*. *Plant Cell Tissue and Organ Culture* **1**, 85-92

Goonatilake, I D (1989). Tissue culture of *Hevea brasiliensis* (seedling shoot tip culture). MSc thesis submitted to the University of Sri Jayawardanapura, Sri Lanka.

Imel, M R and Preece, J E (1988). Adventitious shoot formation from recultured leaves of rhododendron. *Hort Science* **23**, 760 (Abstr).

Kerns, H R and Meyer, M M (1985). *In vitro* propagation of red-silver hybrid maples. *Hort Science* **20**, 593 (Abstr).

Kerns, H R and Meyer, M M (1986). Tissue culture propagation of Acer x freemani using thidiazuron to stimulate shoot tip proliferation. *Hort Science* **21**, 1209-1210

Lloyd, G and McCown, B (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. *Proceedings of international plant Propagators Society* **30**, 421-427.

Mok, M C, Mok, D W S, Armstrong, D J, Shudo, K, Isogai, Y, and Okamoto, J (1982). Cytokinin activity of N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron). *Phytochemistry* **21**, 1509-1511

Nieuwkerk, K V, Zimmerman, R H and Fordham, I (1986). Thidiazuron stimulation of apple shoot proliferation *in vitro*. *Hort Science* **21**, 516-518.

Preece, J E, Huetteman, C A, Ashby, W C, Bresnan, D F and Roth, P L (1988). Provenance tests for biomass production using micropropagated clonal silver maple. *Hort Science* **23**, 803(Abstr).

Singha, S and Bhatia, S K (1988). Shoot proliferation of pear cultures on medium containing thidiazuron and benzyl amino purine. *Hort Science* **23**, 147.

Sipes, D L and Einset, J W (1983). Cytokinin stimulation of abscission in lemon pistil explants. *Journal of Plant Growth Regulation* **2**,73-80.

Sudarsono and Goldy, R G (1991). Growth regulator and axillary bud position effects on *in vitro* establishment of *Vitis rotundifolia*. *Hort Science* **26**, 304-307.

Suttle, J C (1985). Involvement of ethylene in the action of the cotton defoliant thidiazuron. *Plant Physiology* **78**, 272-276.

Swartz, H J (1988). *In vitro* strategies for improving *Rubus* in warm climates. In: *Les Strategies De Selection Face aux Technologies Modernes* (pp 66). Moet-Hennessy Conference, Moet-Hennessy, Paris, France

Thomas, J C and Katterman (1986). Cytokinin activity induced by thidiazuron. *Plant Physiology* **81**, 681-683.

Wang, S Y, Steffens, G L and Faust, M (1986). Breaking bud dormancy in apple with a plant bioregulator, thidiazuron. *Phytochemistry* **25**, 311-317.

(Received 29 July 1994)