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EFFECT OF CYTOKININS ON *IN VITRO* PROPAGATION OF *UAPACA KIRKIANA*

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CHISHIMBA, W. K., LINGUMBWANGA, E., TEMBO, L. M. & HANG'ANDU, A. K. 2000. Effect of cytokinins on *in vitro* propagation of *Uapaca kirkiana*. The effect of five different types of cytokinins, namely N^6-benzyladenine (BA), kinetin, N^6-(2-isopentenyl) adenine (2iP), thidiazuron (TDZ) and zeatin on *in vitro* propagation of *Uapaca kirkiana* was studied using woody plant medium (WPM). Good quality shoots (with a mean of 3.4 shoots per explant) were regenerated on the WPM supplemented with 0.5μ M BA. The suitability of different cytokinins for *in vitro* propagation of *Uapaca kirkiana* was established in the order of BA>TDZ>2iP>kinetin>zeatin. On TDZ-supplemented medium, stunted shoots with comparatively thicker leaves were regenerated. Shoots produced on WPM supplemented with BA rooted easily. *In vitro* regenerated plantlets were established under *ex vitro* conditions.

Key words: Cytokinins - micropropagation - thidiazuron (TDZ) - *Uapaca kirkiana* - wild fruit tree

CHISHIMBA, W. K., LINGUMBWANGA, E., TEMBO, L. M. & HANG'ANDU, A. K. 2000. Kesan sitokinin ke atas pembiakan *in vitro* bagi *Uapaca kirkiana*. Kesan lima jenis sitokinin yang berbeza, iaitu N^6-benziladenina (BA), kinetin, N^6-(2-isopentenil) adenina (2iP), tidiazuron (TDZ) dan zeatin ke atas percambahan *in vitro* bagi *Uapaca kirkiana* dikaji menggunakan media tumbuhan berkayu (WPM). Pucuk yang bermutu tinggi (dengan purata sebanyak 3.4 pucuk setiap eksplan) dipulihkan dengan WPM yang dilengkapkan dengan 0.5 μMBA. Kesesuaian sitokinin yang berbeza bagi percambahan in vitro bagi *Uapaca kirkiana* ditubuhkan dengan turutan BA>TDZ>2iP>kinetin>zeatin. Pucuk terbantut pada media yang dilengkapkan dengan TDZ dengan daun yang lebih tebal secara perbandingan telah dipulihkan. Pucuk yang dihasilkan dengan WPM yang dilengkapkan dengan BA mengakar dengan senang. Anak tumbuhan yang dipulihkan dengan *in vitro* ditubuhkan di bawah keadaan *ex vitro*.

Introduction

Uapaca kirkiana Müll. Arg. is a dioecious wild loquat indigenous to Central and Southern Africa (White 1962, Storrs 1979). It is a semi-deciduous fruit tree belonging to the family Euphorbiaceae. The fruit of *Uapaca kirkiana* is rich in sugars and vitamin C (Sufi & Kaputo 1977). Although the species is still wild, it possesses great economic potential as a pomological tree.

Uapaca kirkiana is difficult to propagate from cuttings but it establishes readily when inoculated with mycorrhizal fungi (Mwamba *et al.* 1992). It has been reported that members of the Euphorbiaceae such as succulent *Euphorbia flanaganii* tend to be difficult to propagate from seed or cuttings (Silberstein *et al.* 1983). Though sexual propagation is possible in *U. kirkiana*, it is not recommended in breeding fruit trees where retention of the appropriate traits is required. Micropropagation techniques can be applied in rapid multiplication of elite trees identified for breeding.

There is no report on the *in vitro* propagation of *U. kirkiana*. However, tissue culture was reported in species of the genus *Euphorbia*. Tideman and Hawker (1982) have reported some work in tissue culture of four latex-producing species, namely *Euphorbia lathyris*, *Euphorbia peplus*, *Asclepias rotundifolia* and *Araujia sericofera*.

Compared to kinetin, N^6-benzyladenine (BA) and some other adenine analogues have proved to be most useful in tissue culture of many woody species (Driver & Kuniyuki 1984, Nieuwkerk & Zimmerman 1986, Zhang *et al.* 1987). Previous research has indicated that there are strong genotypic effects on shoot multiplication *in vitro* (Read 1988). Ripley and Preece (1986) working with *Euphorbia lathyris* found that when the level of BA was increased, apical shoot growth decreased.

Isopentenyl-adenine (2iP) has also been employed in micropropagation because it is less expensive. Like other cytokinins, 2iP enhances shoot production by antagonising apical dominance, resulting in axillary bud break from the cultured shoot tips (Economou & Read 1984). In *Kalmia latifolia*, Lloyd and McCown (1980) found that on lower levels (2 and 4 μM) of 2iP, fewer but larger shoots were regenerated and higher levels (16, 32 and 64 μM) of 2iP caused stunted shoots.

Zeatin is a very expensive cytokinin compared to BA, kinetin and 2iP, but it may be the only one effective in inducing a higher number of shoots and sustaining growth in some species. Zhang *et al.* (1987) in their work on *Euphorbia fulgens* found that an increase in zeatin concentration resulted in a corresponding increase in shoot production. However, shoot length had a negative correlation with zeatin concentration.

Thidiazuron (TDZ) has been receiving much attention after the discovery of its cytokinin-like properties (Kerns & Meyer 1986, Nieuwkerk & Zimmerman 1986, Fellman *et al.* 1987, Huetteman & Preece 1993). Its effects on plantlets *in vitro* include induction of compactness in shoots and leaf thickness. It also induces organogenesis and embryogenesis depending upon the species (Fasolo *et al.* 1989, Huetteman & Preece 1993). The objective of this study was to determine the effect of BA, kinetin, 2iP, TDZ and zeatin on *in vitro* performance of *U. kirkiana* in order to estimate their potential use in rapid micropropagation for domestication purposes.

Materials and methods

Establishment of in vitro culture

Uapaca kirkiana seeds were collected from wild plants in Serenje (Zambia) and sown in pots filled with perlite. Seedlings were maintained in a greenhouse and watered regularly for four months. Shoot tip explants (15 mm) were excised from seedlings, pre-washed with running tap water for one hour and then treated for 10 seconds in 70% ethanol. The shoot tips were removed, rinsed with autoclaved water and disinfested for 20 min on a shaker with 30% sodium hypochlorite (3% available chlorine) containing 0.1% dish-washing detergent as a surfactant. Rinsing was done several times in sterile distilled water under the laminar flow hood. Shoot tips were trimmed to 10 mm and clean cultures established on woody plant medium (WPM) (Lloyd & McCown 1980), supplemented with 2.0 μM BA. These cultures served as an *in vitro* stock for all tissue culture manipulations. The *in vitro* stock was subcultured every six or seven weeks alternately on WPM with BA or WPM without any plant growth regulator (PGR). Explants were derived from seedlings as an initial step in technique development.

In vitro multiplication of culture

Explants containing four nodes (without shoot tips), and previously growing on PGR-free medium, from an established *in vitro* stock were trimmed and cultured on WPM supplemented with one of the following PGRs: BA, kinetin, 2iP, TDZ or zeatin. Unless otherwise stated, the explants were placed on the media vertically with the basal end in contact with the medium. Actual concentrations applied in each cytokinin were BA (0, 0.5, 2.5, 5, 10, 20 or 40 μM); kinetin (0, 0.5, 5, 10, 20 or 40 μM); 2iP (0, 0.5, 2.5, 5, 10, 20, 40, 80, 120 or 160 μM); TDZ (0, 0.5, 5, 10, 20, 40 or 80 μM); and zeatin (0, 0.5, 5, 10, 20, 40 or 80 μM). The basic medium was WPM with Difco Bacto-agar at 7 g l^{-1} and 20 g l^{-1} sucrose. The pH of the media was adjusted to 5.8 with 1M NaOH or 1M HCl prior to the addition of agar, and slight heating. Medium aliquots of 10 ml each were dispensed into 25 × 150 mm borosilicate culture tubes, and autoclaved at 121°C and 1 kg cm^{-2} for 20 min.

Cultures were maintained at 27 ± 2 °C under 16 h per day photoperiod and photosynthetic photon flux provided by cool white fluorescent lamps. Cultures were placed 12 cm below two fluorescent tubes. Due to lack of appropriate gadget, light intensity was not measured. The experimental layout was a completely randomised design (CRD) with 20 explant replications per treatment and was conducted twice.

Rooting of in vitro produced shoots

Rooting was achieved by first growing the plantlets from cytokinin treatments on PGR-free WPM for seven weeks and subculturing on half-strength WPM

supplemented with 0.5 μM indolebutyric acid (IBA). Only those explants from cytokinin treatments with highest number of shoots per explant were utilised in a rooting experiment. Rooted *in vitro* plantlets were potted in sterilised woodland loamy soil with some treatments being maintained *ex vitro* up to five months.

Evaluations that included number of shoots (≥ 5 mm) and roots (≥ 5 mm), callus formation and shoot quality based on vigour were carried out after seven weeks. In most treatments callus was formed at the base of the plantlet. Shoot quality rating was scored on a scale 1.0–3.0 with 3.0 referring to best shoots formed in terms of vigorous appearance, leaf development, colour and ease of handling (Economou & Read 1984).

Data analysis

Statistical analysis on the number of shoots was performed as a one-way classification analysis of variance with unequal replication per treatment. Multiple comparisons were made using LSD to discriminate between means. The statistical package employed was the Montana State University Statistical Analysis package (MSUSTAT) (Lund 1986). The plots were done using Lotus software (1991).

Results

The number of shoots regenerated per explant depended on the nature of cytokinin and the concentration applied. Data presented in Figure 1 indicate that kinetin and zeatin (except at 0.5 μM concentration) inhibit axillary shoot growth compared to the control. As the concentration of kinetin and zeatin increased, the number of shoots regenerated per explant declined, and at higher levels no shoot induction was achieved. Generally, explants on media supplemented with either kinetin or zeatin tended to be sluggish in response. None of the seven 2iP treatments were statistically different from the control. Normally a significant verdict could have been recorded but this was not possible because of considerable variability in the data on a small sample, as the error term remained large.

Shoot proliferation with BA was most effective at lower concentrations. At concentrations as low as 0.5 μM a mean of 3.4 shoots per explant was regenerated (Figure 1). However, when the concentration of BA was increased to 5.0 μM the number of shoots induced decreased by 50%. The most effective BA concentration levels in both shoot proliferation and quality was 0.5 and 2.5 μM, at which signs of effective shoot formation were noted two weeks after culture. Initiation of callus was observed after two weeks at the base of the explant.

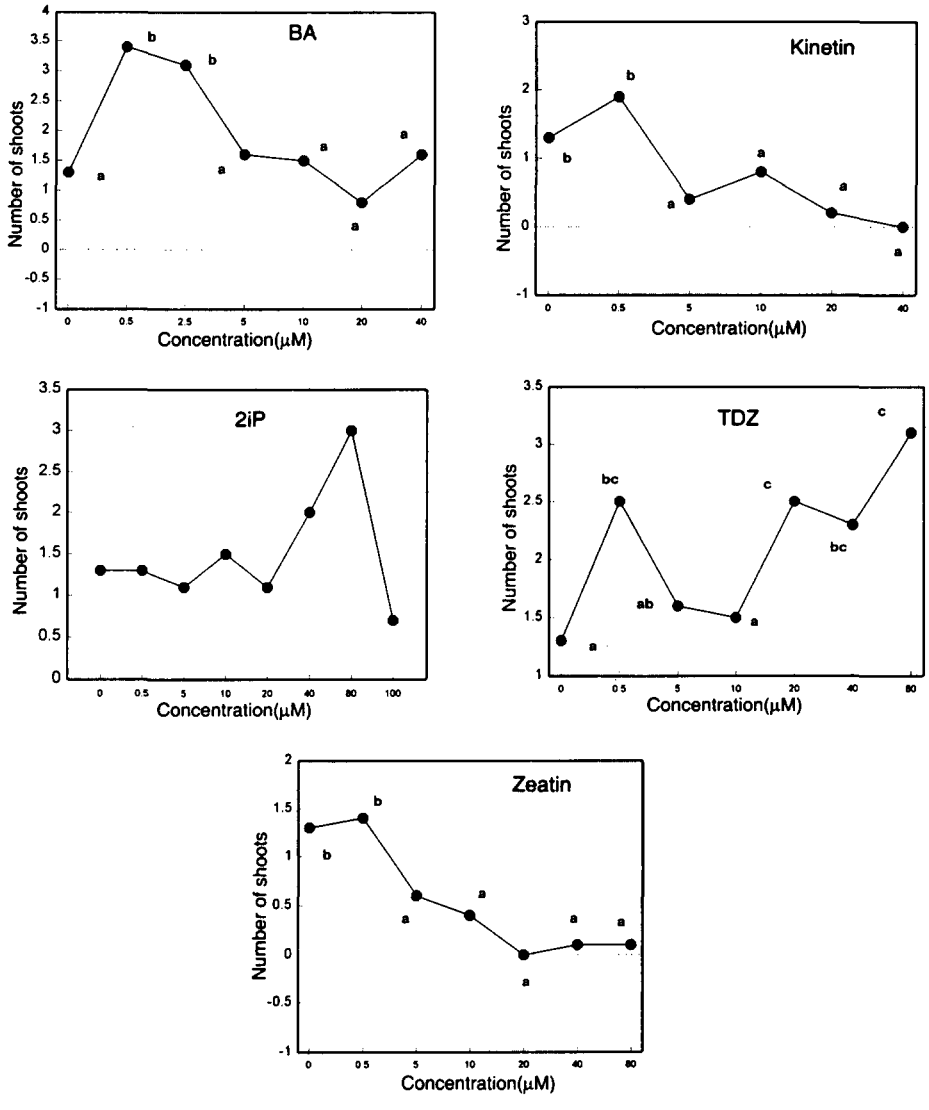

Figure 1. Effect of different cytokinins on *in vitro* shoot proliferation of *Uapaca kirkiana*. Letters indicate LSD at $p < 0.05$.

Thidiazuron was equally effective in regenerating a high number of shoots at all the concentrations that were applied particularly at 80 μM, and by two weeks 50% of the explants had produced shoots. Shoots regenerated by TDZ were characteristically stunted and compact, with thick leaves. The shoots regenerated on media supplemented with TDZ were comparatively inferior in quality to those of BA-supplemented media.

Except for media supplemented with various concentrations of TDZ, on all the other cytokinins, regenerated lateral shoots were characterised by apical dominance in the main shoot. With TDZ, newly induced shoots originated from the base of the explant. On higher TDZ concentrations, adventitious shoot formation was more prevalent.

Shoot proliferation as a measure of cytokinin effectiveness on *Uapaca kirkiana* decreased in the following order: BA>TDZ>2iP>kinetin>zeatin (Figure 2). Generally, a low amount of callus was recorded in the majority of the treatments (data not included).

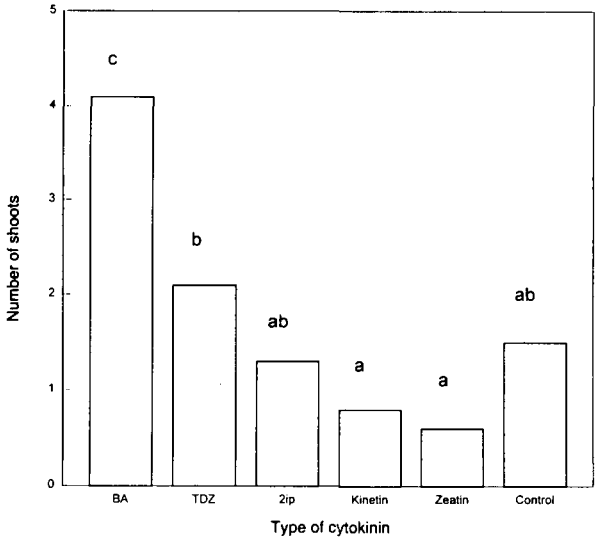

Figure 2. Total shoot proliferation per cytokinin as a measure of cytokinin effectiveness on *Uapaca kirkiana*. Letters indicate LSD at $p<0.05$.

In vitro rooting was comparatively better on plantlets previously cultured on media supplemented with BA (80%); and poor on media supplemented with kinetin (20%) (Table 1). Plantlets were established *ex vitro* for four months in pots before they died due to crude acclimatisation facilities and lack of mycorrhizal inoculum which is essential for root establishment in *Uapaca kirkiana*. At least six plantlets from 2.5 µM BA treatment level survived up to five months (Figure 3).

Table 1. Influence of cytokinin on *in vitro* rooting of *Uapaca kirkiana*

Cytokinin	Mean number	Percentage of rooted plantlets	n
Control	1.25 ± 0.26	75	10
Kinetin	1.33 ± 0.21	20	3
2iP	1.63 ± 0.45	60	6
BA	1.94 ± 0.16	80	10
TDZ	1.60 ± 0.14	70	7
Zeatin	1.75 ± 0.18	40	4

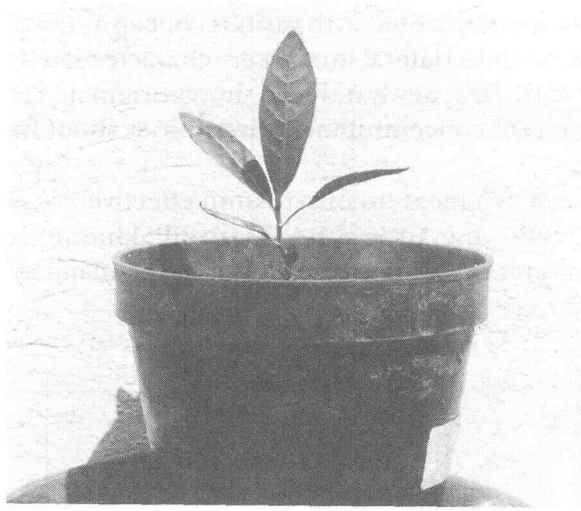

Figure 3. *In vitro Uapaca kirkiana* from WPM supplemented with 2.5 μM BA maintained *ex vitro* up to five months

Discussion

The achievement of *in vitro* shoot production of up to three shoots or more per explant on WPM supplemented with BA, TDZ or 2iP demonstrates further the broad effectiveness of these cytokinins in various plant species (Figure 1). The three cytokinins have been widely used in tissue culture of many plant species, particularly woody species, each with varying degrees of success or effectiveness. In this study, BA proved to be the best cytokinin for *in vitro* propagation of *U. kirkiana* in terms of number of shoots regenerated per explant, favourable effect on rooting and the quality of such shoots. Benzyladenine is an effective cytokinin for inducing axillary shoot proliferation (Ripley & Preece 1986) at optimal concentration levels. Concentrations above optimum tend to inhibit further axillary shoot development. The other advantage with BA when compared to the other four cytokinins is that maximum shoot regeneration was achieved at concentrations as low as 0.5 μM–2.5 μM (Figure 1). This means BA would be cheaper to apply in micropropagation of *U. kirkiana.* Conversely, kinetin and zeatin particularly, were not as good as the other three PGRs and hence initiated the least number of shoots per explant (Figure 1). Generally, kinetin has not been widely used in tissue culture of woody species particularly with the advent of other cytokinins, because of apparent weakness. Furthermore, the *in vitro* response of any plant to cytokinins is genotypically dependent (Read 1988). However, zeatin has been applied in a number of cases and the effectiveness in shoot proliferation has been variable depending on the genotype and the physiology of the plant species. Apparently, higher kinetin, 2iP and zeatin concentration levels might have had toxic effects on *U. kirkiana* because in some treatments, explants died. The number of dead explants was

highest at 40 μM kinetin, 160 μM 2iP and 80 μM zeatin where the percentage losses were 55, 60 and 50 respectively (data not included). The effect of zeatin was negative on *in vitro* culture of *U. kirkiana* although it has proved to be very potent on other plants such as *Euphorbia fulgens* (Zhang *et al.* 1987).

The quality of shoots produced on media supplemented with TDZ was not very good. The shoots were stunted with shorter internodes and thickened leaves, an observation consistent with the effects of TDZ (Fasolo *et al.* 1989). This is in contrast with the quality of shoots arising from BA-supplemented medium (Figure 3).

Apart from kinetin, the effect of various cytokinins on rooting was not apparent. This is very good because it means shoots regenerated *in vitro* can be easily rooted without inhibition due to residual cytokinins. It was, however, noted that *ex vitro* establishment of the plantlets was very difficult resulting in many plants dying. This can be attributed to lack of sterile mycorrhizal inoculum at the time of transplanting. Work done by Mwamba (1995) established that a mixture of mycorrhizal isolates and a selection of pure isolates significantly stimulated new root growth and enhanced seedling survival. Plantlets produced through an *in vitro* system will certainly require an inoculum of root-inhabiting fungi, for enhanced robustness *ex vitro* and successful domestication.

Conclusion

The results demonstrated that BA is the most suitable cytokinin for *in vitro* propagation of *U. kirkiana.* Benzyladenine induced the maximum number of good quality shoots per explant. The effect of BA on *in vitro* rooting was favourable and yielded more rooted plantlets than the other four cytokinins.

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