Journal of Medicinal Plants Research Vol. 5(1), pp. 1-6, 4 January, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Propagation and chlorophyll fluorescence of *Camptotheca acuminata* cuttings

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Accepted 15 September, 2010

The Chinese happy tree, *Camptotheca acuminata* Decne, is grown extensively in plantations for harvest and extraction of its anti-cancer and potentially anti-viral compound, camptothecin. This study determined whether *C. acuminata* was amenable to clonal propagation as rooted cuttings and whether application of the rooting hormone, indole-3-butyric acid (IBA), affected chlorophyll fluorescence (F_V/F_M) or the percentage of cuttings that formed roots. The species was found highly amenable to vegetative propagation because a consistently high percentage of cuttings formed roots when treated with 3 or 8 g IBA / kg powder. IBA application was unnecessary in spring when more than 90% of untreated cuttings formed roots, but the highest IBA dose increased the number of plants produced by 18 and 82% (from tip cuttings) and by 46 and 102% (from node cuttings) in two later collections when rooting of untreated cuttings had declined. IBA treatments did not affect chlorophyll fluorescence. Industrial deployment of *C. acuminata* is entirely feasible using rooted cuttings, allowing clonal multiplication of genotypes that contain high levels of camptothecin.

Key words: Adventitious roots, auxin, *Camptotheca acuminata*, camptothecin, photoinhibition, propagation

INTRODUCTION

The Chinese happy tree or *Xi Shu* (*Camptotheca acuminata* Decne, Cornaceae) is an endangered species from southern China (Li and Adair, 1994; Liu and Adams, 1998; Liu et al., 2002) that is grown extensively as a plantation tree for extraction of its anti-cancer compound, camptothecin (Li and Adair, 1994; Li and Liu, 2003; Lorence and Nessler, 2004). Camptothecin and its derivatives, such as irinotecan and topotecan, are used for the treatment of colorectal, ovarian, cervical and lung cancers, and have potential for treatment of a wide range of viruses and parasites (Lorence and Nessler, 2004; Basili and Moro, 2009). The species has been grown from seed (Li and Adair, 1994; Liu et al., 1999, 2002; Maxwell, 2003; Pasqua et al., 2004) and there are many reports describing in vitro methods for clonal propagation, including shoot culture (Jain and Nessler, 1996; Wiedenfeld et al., 1997; Liu and Li, 2001), shoot regeneration from hypocotyl, leaf or petiole callus (Wiedenfeld et al., 1997; Li and Liu, 2005; Wang et al., 2006) and somatic embryogenesis (Sankar-Thomas et al., 2008).

Clonal propagation, either in vitro or by rooted cuttings, has the potential to maximise yield, quality and uniformity following selection of desired genotypes and it can overcome limitations to domestication for species, such as C. acuminata, that are extremely rare or have limited seed availability (Leakey et al., 1994; Trueman et al., 2007). There are few reports describing propagation of C. acuminata leaf or stem cuttings. Li and Adair (1994) indicated that the species can be propagated from leafy cuttings, Liu and Adams (1996) stated that their assay plants had been propagated from branch cuttings of a mature tree, and Maxwell (2003) reported that root formation on cuttings from 3-year-old trees was greatly increased by application of the auxin, indole-3-butyric acid (IBA).

IBA application is one of the most common and possibly most effective methods to enhance root formation in cuttings (Blazich, 1988a; Hartmann et al., 1997; Leakey, 2004). IBA increases the percentage of cuttings that forms roots in a wide range of trees and shrubs (Henrique et al., 2006; Husen and Pal, 2007; Ali

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et al., 2008; Holloway et al., 2008; Husen, 2008; Krisantini et al., 2009; Vakouftsis et al., 2009) but cuttings of some species appear unresponsive to auxin (Shiembo et al., 1996; Atangana et al., 2006; Trueman and Peters, 2006; Araya et al., 2007) and high auxin doses can cause cutting death (Perry and Trueman, 1999; Zuffellato-Ribas and Rodrigues, 2001; Wendling and Xavier, 2005; Trueman and Richardson, 2008). Cuttings must survive physiological stress after severance from the stock plant, with little water or nutrient uptake until roots penetrate the propagation medium (Grange and Loach, 1983; Blazich, 1988b; Hartmann et al., 1997). During this period, leaves of cuttings may be prone to reductions in the maximum photochemical efficiency of photosystem II; i.e. reduced chlorophyll fluorescence (F_V/F_M) (Mesén et al., 1997, 2001; Bruce et al., 2001; Pohio et al., 2005; Trueman and Richardson, 2008; Misra and Misra, 2010). Little is known about the effects of applied auxins on chlorophyll fluorescence of cuttings despite their widespread use in cuttings propagation systems and the potential for high auxin doses to induce physiological stress (Trueman and Richardson, 2008).

The objective of this study was to determine whether *C. acuminata* is amenable to clonal propagation as rooted cuttings, and specifically whether (1) a high percentage of cuttings forms roots, (2) IBA causes leaf stress, evident as reduced F_V/F_M; and (3) IBA affects the percentage of cuttings that forms roots. These results would assist in developing a nursery propagation system for *C. acuminata* industrial deployment.

MATERIALS AND METHODS

Seeds of *C. acuminata*, obtained from B and T World Seeds (Paguignan, France), were sown between January and May 2008 in 220 ml propagation tubes containing potting mix with a thin covering of vermiculite, and germinated under mist irrigation in a glasshouse in Gympie (26°09'S, 152°38'E), Australia. Seedlings were transplanted between July and September 2008 into 4 L pots containing a 75/25 (v/v) mix of shredded pine bark and perlite with 3 kg of 8 to 9 month slow release Osmocote™ fertiliser (Scotts International, Heerlen, The Netherlands), 3 kg of lime (Unimin, Lilydale, Australia), 1 kg of gypsum (Queensland Organics, Narangba, Australia), 1 kg of Micromax ™ micronutrients and 1 kg of Hydraflo2 ™ wetting agent (both from Scotts Australia, Baulkham Hills, Australia) incorporated per m^3. Pots were immediately transferred to a translucent white polyethylene chamber. Thirty-one seedlings were established, and these were pruned commencing in May 2008 to create hedged stock plants with a minimum height of 25 cm (Figure 1A). Rooted cuttings from these stock plants were also established between May and July 2008 in 220 ml propagation tubes and, subsequently, 4 L pots so that the ortet and two ramets were maintained as stock plants for each clone.

Shoot tip cuttings, comprising the distal 5 cm of the main stem or vertically-oriented branches, were collected from the stock plants and inserted into propagation medium on three occasions: 14 October 2008 (spring), 25 November 2008 (early summer) and 26 February 2009 (late summer). Node cuttings, comprising 5 cm nodal segments of vertically-oriented branches, were collected and inserted on the same occasions. Cuttings were pruned prior to insertion by removing half to two-thirds of the length of each expanded leaf. Two tip cuttings and two node cuttings were collected from each of 20 clones on the last two collection dates, pruned, dried at 65°C, and weighed. Twenty other cuttings, when available, were collected from each clone and allocated randomly to each of four hormone treatments: 0, 1, 3, or 8 g IBA / kg talcum powder. Cuttings were dipped 0.5 cm into treatment powder for about 1 s and placed 1 cm deep in a 90 cm^3 tube containing a 50/50 (v/v) mix of perlite and shredded pine bark with 3 kg of 8 to 9 month slow release Osmocote ™ fertiliser (Scotts International, Heerlen, The Netherlands) and 1 kg of gypsum (Queensland Organics, Narangba, Australia) incorporated per m^3. Tubes were then placed under mist irrigation in an adjacent glasshouse. Misting was provided for 10 s every 10 min from 0600 to 1800 H and for 10 s every 20 min from 1800 to 0600 H. Total numbers of tip and node cuttings, respectively, were 157 and 191 (spring), 198 and 362 (early summer) and 309 and 232 (late summer).

Chlorophyll fluorescence (F_V/F_M) of tip cuttings was measured during the early summer and late summer experiments. Fluorescence was recorded at 1100 H from adaxial leaf surfaces of 15 cuttings per treatment at 1 , 9 , 19 , 27 and 40 day post-insertion. Different cuttings were measured on each occasion. Leaves were dark adapted for 15 min, and fluorescence values were obtained with a 5 s flash of 80% of the available light (approx. 2400 µmol photons m^{-2} s^{-1}) using a Fluorescence Induction Monitor (Analytical Development Co. Ltd, Hoddesdon, UK). These settings, determined from preliminary tests on a previous sample of cuttings, were kept constant for all measurements. Irradiance was determined using a quantum sensor (Delta-T Devices Ltd, Cambridge, UK) when chlorophyll fluorescence of cuttings was recorded. Temperatures were recorded for the duration of the last two experiments using Tinytag extra data loggers (Hastings Data Loggers, Port Macquarie, Australia).

Presence or absence of roots was examined 42 d after insertion into propagation medium. The rooted cuttings were required for subsequent pot and field assessments of camptothecin accumulation (Figure 1B) and so root number, root length, and timing of root initiation were not determined.

Dry weights of tip and node cuttings were compared using paired t-tests. Chlorophyll fluorescence was analysed by 2-way ANOVA (IBA treatment × day) because significant interactions were not detected between these two factors in either early or late summer. Proportions of tip and node cuttings that formed roots following each IBA treatment were compared with control proportions using chi-squared tests, with significance test values adjusted using sequential Dunn–Šidák corrections. Dry weight and fluorescence means are reported with standard errors, and treatment differences or interactions were regarded as significant at $P < 0.05$.

RESULTS

Dry weights of *C. acuminata* tip cuttings (174.0 ± 8.1 and 362.9 ± 34.4 mg) were higher than those of node cuttings (107.0 ± 7.3 and 213.1 ± 18.5 mg) in both the early and late summer collections, respectively. Irradiances in the glasshouse at 1100 H at 1, 9, 19, 27 and 40 days post-insertion, respectively were: 232, 430, 885, 835 and 253 µmol m^2 s^{-1} (early summer collection) and 687, 698, 436, 348 and 194 µmol m^2 s^{-1} (late summer collection). All of these days were cloudy except for 19 and 27 days after insertion in early summer. Glasshouse temperatures ranged between 16.6 and 35.4°C (Figures 2A and B).

Chlorophyll fluorescence (F_V/F_M) was high on the day after insertion and remained high (approx. 0.80) at 9 days

Figure 1. (A) Stock plant of *C. acuminata* with seven shoots suitable for harvest of shoot tip and node cuttings (scale bar = 10 cm); and (B) rooted cuttings of *C. acuminata* at 1-year post-insertion (scale bar = 25 cm).

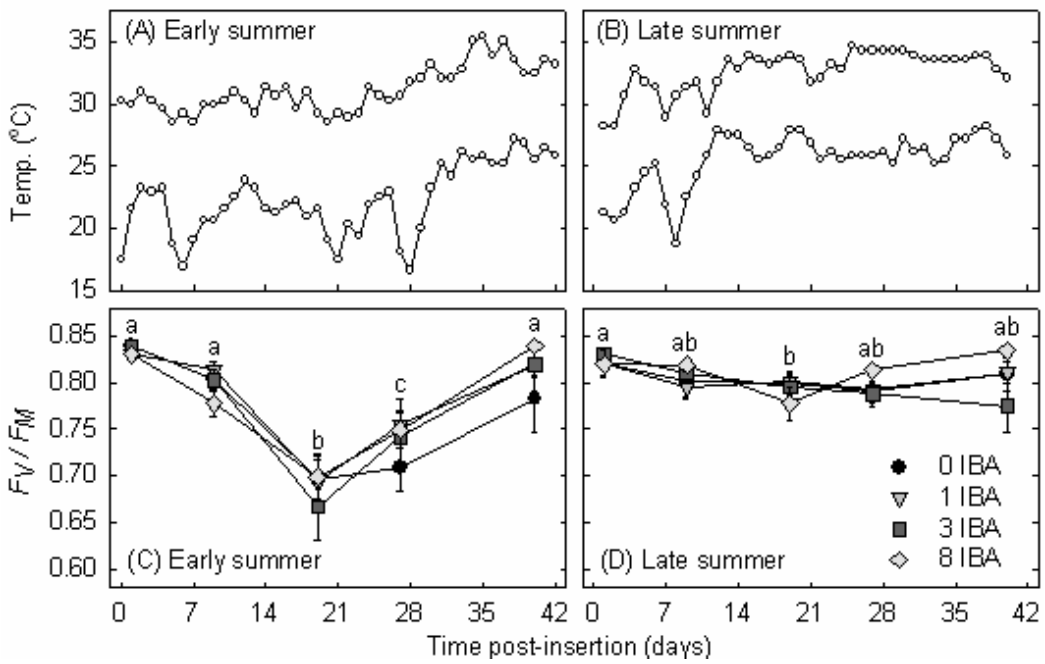

Figure 2. Daily maximum and minimum glasshouse temperatures following collection of *C. acuminata* shoot tip and node cuttings in early summer (A) or late summer (B); and maximum photochemical efficiency (F_V/F_M) at 1100 H for shoot tip cuttings treated with one of four levels of rooting hormone (0, 1, 3 or 8 g indole-3-butyric acid / kg powder) in early summer (C) or late summer (D). F_V/F_M means are provided with standard errors (n = 15). Treatment effects and interactions are not significant, but days with different letters have significantly different means (2-way ANOVA and Tukey's HSD test, $P < 0.05$).

after insertion (Figures 2C and D). F_V/F_M declined significantly by 19 and 27 days post-insertion in early summer and, to a lesser extent by 19 d post-insertion in late summer, but it recovered by 40 days. IBA treatments had no significant effect on F_V/F_M.

In contrast with the lack of effect on chlorophyll fluorescence, IBA application often affected the percentage of cuttings that formed roots (Figure 3). All untreated tip cuttings and 90.2% of untreated node cuttings formed roots in the spring experiment, and IBA application had no significant effect on rooting. However, only 82.8 and 47.1% of untreated tip cuttings and 61.5 and 32.7% of untreated node cuttings formed roots in the early and late summer experiments, respectively. In these experiments, one or both of the higher IBA doses significantly increased rooting. Percentages of tip cuttings that formed roots following the 8 g IBA / kg treatment were 97.7 and 85.9%. Percentages of node cuttings that

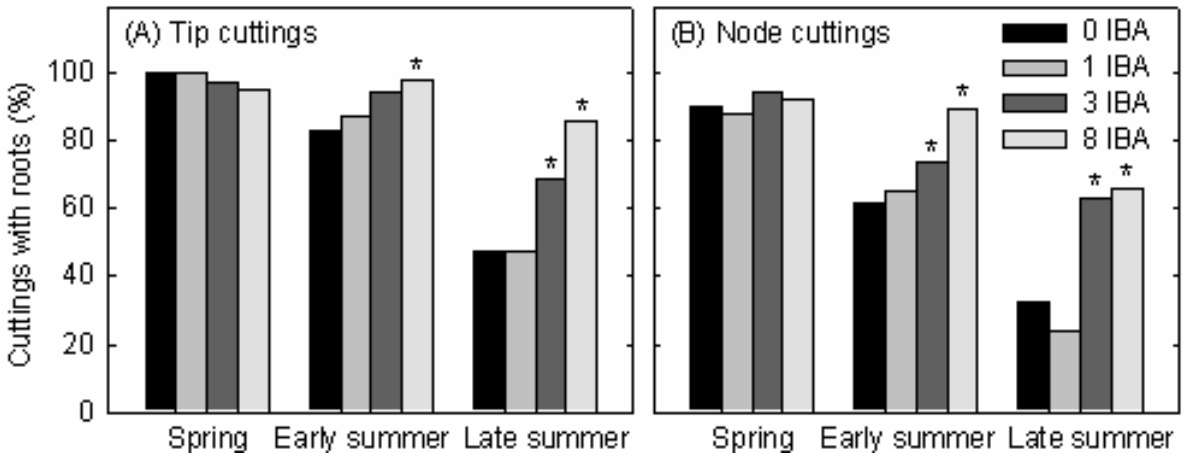

Figure 3. Percentage of *C. acuminata* shoot tip cuttings (A) and node cuttings (B) with roots after treatment with one of four levels of rooting hormone (0, 1, 3 or 8 g indole-3-butyric acid / kg powder) in spring, early summer or late summer. Treatment means with an asterisk (*) are significantly different from their control mean (chi-squared test, $P < 0.05$, n = 35-95).

formed roots following the same treatment were 89.5 and 66.1%. These represented relative increases in plant production of 18 and 82% for tip cuttings and 46 and 102% for node cuttings for the early and late summer collections, respectively.

DISCUSSION

C. acuminata proved highly amenable to propagation as rooted cuttings because a consistently high percentage of cuttings formed roots when cuttings were treated with the higher IBA doses (3 or 8 g IBA / kg powder). IBA application was unnecessary in spring, when all untreated tip cuttings and approximately 90% of node cuttings formed roots, but high IBA doses ensured high rooting percentages in summer when the proportion of untreated cuttings that formed roots had declined. These results confirm that auxins can be most beneficial for increasing rooting in more difficult-to-root cuttings (Blazich, 1988a; Hinesley and Snelling, 1997; Stubbs et al., 1997; Brennan and Mudge, 1998; Wendling et al., 2000) but may provide no benefit when rooting percentages are already high (Stein et al., 1990; Hinesley and Snelling, 1997; Trueman and Peters, 2006). However, because rooting may vary from season to season and from collection to collection, IBA application is recommended for consistently high rooting of *C. acuminata* cuttings.

IBA application had no effect on chlorophyll fluorescence. *C. acuminata* cuttings experienced little or no photoinhibition for the first 9 days after severance from the stock plant and placement under mist irrigation. Maximum photochemical efficiency (F_V/F_M) of cuttings remained high during this phase, at about 0.80. F_V/F_M under optimal conditions is approximately 0.83 for most species, with decreasing values indicating increasing exposure to stress (Björkman and Demmig, 1987; Maxwell and Johnson, 2000). Similarly-high F_V/F_M values have been observed for cuttings of *Wollemia nobilis* (Pohio et al., 2005), *Corymbia torelliana*, *Corymbia citriodora* and *Corymbia torelliana* × *Corymbia citriodora* (Trueman and Richardson, 2008) during the first two weeks under intermittent mist, whereas F_V/F_M fell rapidly for cuttings of *Cordia alliodora* (Mesén et al., 1997), *Albizia guachapele* (Mesén et al., 2001) and *Taxus* × *media* (Bruce et al., 2001) in non-mist propagation systems. In combination, these results indicate that mist irrigation minimizes vapour pressure deficit and prevents rapid onset of leaf stress following severance of cuttings from the stock plant.

Reductions in chlorophyll fluorescence of *Camptotheca* cuttings became more evident by 19 days after severance in early summer (0.67 to 0.70) and, to a much lesser extent, in late summer (0.78 to 0.80). The two occasions that provided lowest F_V/F_M (19 and 27 days post-insertion in early summer) were the two days with highest irradiance (885 and 835 μmol m^2 s^{-1}); however, cuttings displayed high F_V/F_M on two other days (1 and 9 days post-insertion in late summer) that also had high irradiance (687 and 698 μmol m^2 s^{-1}). The early summer values (0.67 to 0.70) were similar to F_V/F_M reported for *Wollemia* and *Corymbia* cuttings under mist (Pohio et al., 2005; Trueman and Richardson, 2008) but less severe than the physiological stress experienced by cuttings of *Cordia* (0.12 to 0.60) and *Albizia* (0.45 to 0.59) in non-mist propagators (Mesén et al., 1997, 2001). Recovery of F_V/F_M upon formation of roots was reported for cuttings of *Taxus* × *media, Wollemia nobilis* and *Corymbia torelliana* (Bruce et al., 2001; Pohio et al., 2005, Trueman and Richardson, 2008) and it is possible that the recovery of F_V/F_M observed in *C. acuminata* occurred as a result of

root formation and consequent water and nutrient uptake from the propagation medium, regardless of daily irradiance levels.

The ultimate objective of this study was to develop a vegetative propagation system for plantation establishment of *C. acuminata* cuttings. This species proved very easy to propagate as rooted cuttings under a glasshouse misting system. More than 90% of cuttings formed roots in spring, and IBA application ensured that rooting percentages remained high when the percentage of untreated cuttings that formed roots had declined. In these cases, the highest IBA dose increased plant production by 18 and 82% (from tip cuttings) and 46 and 102% (from node cuttings). Such increases can have enormous effects on final numbers of field plants, because each rooted cutting can develop into a stock plant that, in turn, produces more rooted cuttings and then more stock plants (Trueman et al., 2007).

ACKNOWLEDGEMENTS

We thank Dr Craig Davis for advice and assistance, Tracey McMahon, John Oostenbrink, Amanda Kilkenny, Kylee Knight, Laura Simmons and Elektra Grant for technical assistance, and the Rural Industries Research and Development Corporation and Nutratec Pty Ltd for financial support.

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