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Source: Tropical Conservation Science, 13(1)

Published By: SAGE Publishing

URL: https://doi.org/10.1177/1940082920966901

Tropical Conservation Science
Volume 13: 1–8

DOI: 10.1177/1940082920966901
journals.sagepub.com/home/trc
SAGE

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Benjamin E. Deloso, Charles J. Paulino and Thomas E. Marler

Abstract

Improved horticultural practices may help to reduce demand for wild cycads threatened by unsustainable collection. We determined the influences of leaf retention with or without anti-transpirants on the success and speed of adventitious root development of *Zamia furfuracea* L.f. and *Zamia integrifolia* L.f. stem cuttings. Root formation success for both species was greater than 95%. The experimental treatments did not influence the percentage success or the speed of root development for *Z. furfuracea* or *Z. integrifolia*. The ending dry weights of the stems, leaves, and roots were also not influenced by the experimental treatments. Our results indicated that adventitious root formation on stem cuttings of these two *Zamia* species was highly successful with or without retained leaves, and horticultural application of transpiration-reducing products on retained leaves did not improve success. Our findings add to the growing body of evidence that show how the horticulture nursery industry can meet horticultural demands in an effort to stop wild harvesting of threatened plants. Conservation of cycads as a group would benefit from more horticulture studies such as this, especially if the research includes threatened species.

Keywords

asexual propagation, cycad, anti-transpirants, *Zamia furfuracea*, *Zamia integrifolia*, horticulture, conservation

Conservation of threatened plant species has been an ongoing global effort to preserve biodiversity. The term "conservation horticulture" was coined in the 1990s to promote the unique skills and knowledge required by horticulturists to aid in the conservation of threatened plants (Affolter, 1997). Other horticultural avenues of conservation have been explored, including seed production and storage, propagation and tissue culture, mineral nutrition, growth regulation, soil management, and protection from pests and diseases (Affolter, 1997). With the unfortunate downward trend of many plant species in the wild, these activities performed by horticulturists are becoming increasingly crucial for the conservation of threatened plants. Indeed, since the establishment of this sub discipline, the unique abilities of horticulturists to answer conservation questions has been recognized (Marler, 2017).

Cycads are gymnosperms that comprise the most endangered group of plants worldwide, with more than 63% of described taxa listed as threatened (Brummitt et al., 2015; Fragniere et al., 2015). Cycads are a primitive extant group of seed plants whose origins have been traced back to the late Paleozoic era (270–250 mya) and reached their maximum worldwide diversity during the Jurassic era (193–136 mya) (Jones, 1993; Mamay, 1969; Norstog & Nicholls, 1997). Research into this plant

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Received 2 August 2020; Accepted 26 September 2020

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group provides insight on the evolution of seed plants (Brenner et al., 2003).

The popularity of cycad species in the horticulture trade has grown in recent years due to their ancient origins and attractive forms. Although cycad conservation would benefit greatly from horticultural studies, the primary literature on this group has focused on taxonomy and ecology (Cascasan & Marler, 2016). Additionally, cycad horticulture and physiology research that would improve conservation decisions has been lacking, despite calls for more research in these fields (Donaldson, 2003).

The cycad horticulture industry utilizes sexual and asexual propagation methods, and both methods were reviewed with an outline of advantages and disadvantages (Dehgan, 1983, 1999). Many cycad species including many *Zamia* produce adventitious stems especially near the base of the main stem known as “suckers” (Haynes, 2009). Additionally, adventitious stems found above ground level attached to the main stem are known as “offsets” (Haynes, 2009). These “suckers” and “offsets” are also known as “pups” in the nursery trade (Norstog & Nicholls, 1997; Stopes, 1910; Tang, 1985). Detached pups often readily produce adventitious roots (AR) during successful asexual propagation protocols. Additionally, some cycad species such as *Cycas micronesica* K.D. Hill that are adapted to frequent tropical cyclones may form natural vegetative propagules after falling. These fallen stems often form adventitious roots at the points of soil contact (Marler & Cruz, 2017).

Some plant species require leaf retention on stem cuttings to ensure propagation success (Hartmann et al., 1990). Anecdotal recommendations for how to treat existing leaves during cycad propagation protocols are varied. Some recommendations are specific and call for removal of all leaves from cycad stem cuttings (Jones, 1993). Other recommendations are equally specific and call for retention of one or two leaves following removal of the remainder of leaves (Tang, 1985). Some recommendations are less specific and indicate “some” of the existing leaves should be retained on cycad stem cuttings (Whitelock, 2002). Many cycad horticulturists remove all leaves from an established cycad plant before transplanting (Haynes, 2012). This disparity in recommendations is confusing and illuminates the fact that no replicated empirical studies have been conducted to determine how to approach the issue of leaf retention on cycad stem cuttings and whether retaining leaves on cycad stems cuttings confers any benefit.

When leaves are retained on stem cuttings, horticulturists must acknowledge that the cohesion-adhesion column of water that connects stomata to roots and enables transpiration in an intact plant is disrupted by the lack of roots on the cuttings. Water loss from retained leaves must be reduced to avoid desiccation of the cuttings during the propagation phase (Davis et al., 1988). One commercial approach involves intermittent mist systems which periodically re-wet the leaves to reduce water loss (Hartmann et al., 1990). Other approaches have employed anti-transpirant products to minimize water loss from the leaves retained on cuttings (Whitcomb & Davis, 1970). The most common products for this purpose coat the surfaces of leaves and physically block stomatal gas exchange (Baggot & Joiner, 1978; Gur et al., 1986; Lyons et al., 1985; Switras-Meyer & Gillman, 2002; Whitcomb et al., 1974). Other products are substances applied to roots that reportedly increase abscisic acid (ABA) production in roots. If the application of these products precedes excision of cuttings or digging of transplants, increased ABA translocation to leaves may cause stomatal closure in the cuttings (Dabirian & Miles, 2017).

Previous studies on cycad asexual propagation did not utilize replicated trials and more importantly did not include a control group. The first replicated trial investigating adventitious root formation on cycad stem cuttings found that a wide dose range of indole-3-butyric acid (IBA) exhibited little influence on numerous traits, including the speed of initial root formation (Deloso et al., 2020). Additionally, to our knowledge there have been no replicated trials that objectively determined the positives or negatives that result from retaining leaves on cycad stem cuttings. Moreover, there have been no studies on the use of transpiration reducing compounds for any horticultural operation involving cycads. Our primary objective was to determine the influence of leaf retention on the success and speed of adventitious root formation on *Z. furfuracea* and *Z. integrifolia* stem cuttings. Our secondary objective was to evaluate two anti-transpirant products for efficacy of improving adventitious root formation on cuttings with retained leaves.

Methods

Six-year-old seedlings of the Mexican cycad *Z. furfuracea* in 210 mL tubes were used to make stem cuttings at the University of Guam. The experiments were repeated with six-year-old seedlings of *Z. integrifolia*, a related species native to the southeastern United States. These model species were chosen based on local availability, abundance in the nursery trade, and relative ease of horticultural care.

Two anti-transpirant products were selected, one used as a water-soluble compound to be taken up by the roots and the other used as a physical coating on the leaves. The water-soluble product was Root-Drench® (Zorro Technology Inc., Clakamas Oregon, USA) and the physical leaf coating product was Wilt-Pruf® (Wilt-Pruf Products, Inc., Essex, Connecticut, USA).

Plants without noticeable active leaf expansion were used. Each replication consisted of five cuttings for a total of 25 replications per study and 250 stem cuttings total. Cuttings were subjected to one of the following treatments randomly: control with no leaves (no anti-transpirant compounds), control with leaves (no anti-transpirant compounds), Root-Drench only, Wilt-Pruf only, and Root-Drench + Wilt-Pruf. For the control with leaves and the three anti-transpirant treatments, leaves were pruned at the base of each petiole to remove all but two leaves. For the plants receiving the Root-Drench treatment, 20 mL of the product was applied to the subtending soil to each plant two days prior to removal of the roots, then 20 mL was applied again one day prior to removal of the roots in accordance with the manufacturer's directions. On the third day the root system was cut at the base of each stem to remove all root tissue and expose a clean cut. This is an unambiguous action because vascular tissue arrangement differs in stem and root tissues and can be easily observed on the open wound (Marler, Lindström et al., 2010).

For the Wilt-Pruf treatment, the two retained leaves were dipped in the Wilt-Pruf solution then allowed to dry for 24 hours. The plants were then lifted out of their respective containers and the roots were excised as previously described. For the Root-Drench + Wilt-Pruf treatment, plants received both the 40 mL of Root-Drench and leaves were coated in Wilt-Pruf as previously described. The Wilt-Pruf coating was applied on the second day to coincide with the second application of Root-Drench. Afterwards the roots were excised as previously described.

Each stem cutting was first dipped in 10% bleach solution (The Clorox Company, Oakland, California, USA), then in a 0.13% Daconil fungicide solution (Techpac LLC, Atlanta, Georgia, USA), then surface water was allowed to air-dry. The Hormex root stimulant powder (Brooker Chemical Corp., Chatsworth, California, USA) was used as our source of IBA. The cut end of each stem was dipped into 8 $mg \cdot g^{-1}$ IBA.

The exposed parenchyma was then covered with a commercial tree sealant and allowed to air dry for 24 hours. Clear 2.84 L Cambro containers (Cambro Manufacturing Company, Huntington Beach, California, USA) were used for the study. One container was designated as a replication which contained one cutting per treatment. Similarity in stem diameter was used to select the five cuttings for each replication. The containers were prepared with drainage holes drilled at 5.5 cm apart to ensure that each stem cutting was positioned with equal spacing from each other and homogeneous distance to drainage holes.

Perlite (Therm-O-Rock West, Inc, Chandler, Arizona, USA) was filled to the 2.37 L level and each cutting was placed in a diagonal pattern so that each cutting had access to two drainage holes. The cuttings were assigned to each available position in a random manner. After placing the cuttings directly on the perlite, the containers were then filled with additional perlite to the 2.84 L level and watered. To keep the cuttings with retained leaves upright in the rooting medium, a wire frame was constructed around each container. The date of planting for each replication was recorded. The start date for the *Z. furfuracea* study was 04 January 2018, and the start date for the *Z. integrifolia* study was 10 August 2018. Containers were placed under 50% shade, positioned in a Randomized Complete Block design with each container treated as a block, and maintained under plexiglass rain exclusion.

The high and low temperatures were recorded daily throughout the experiment using an AcuRite outdoor thermometer (Chaney Instrument Company, Lake Geneva, Wisconsin, USA) covered with a radiation shield. Containers were watered three times weekly. The transparent bottom surface of the experimental pots was inspected daily until adventitious roots made first contact with the surface. This approach enabled the determination of speed of root formation without disturbing the perlite or cuttings.

Each individual rooted cutting was removed from the perlite at the time of root contact, then was planted into 2.6 L containers filled with a medium consisting of 50% sunshine mix #4: 25% #16 sand: 25% field soil. This medium was comprised of approximately equal volumes of peat, perlite, sand, and soil. Other non-destructive variables were also recorded throughout the duration of the experiment. These included: the date of first leaf emergence, the date of cutting death (if mortality occurred), and the overall appearance of the AR system at the time of removal from perlite. Photographs were used to aid in this endeavor.

The plants were maintained until the final cutting in each experiment produced AR or was identified as dead. Beginning this date, all plants in the study were bare rooted then separated into roots, stems, and leaves. The fresh weight of each organ was recorded, and the number of coralloid root clusters was counted for each plant. Each stem was cut in half with a radial section to reveal the precise location where adventitious roots were initiated at the base of each cutting. Each plant organ was placed into a forced draft oven at 75°C for 48 hours, then the dry weight was recorded.

Statistical analyses were performed using R commander (R package version 2.6-0) (Fox & Bouchet-Valat, 2020). Response variables were subjected to analysis of variance (ANOVA) with each experimental treatment as the single factor. For each significant response variable, a Tukey's post hoc test was used for means separation.

Figure 1. (A) *Zamia furfuracea* stem cutting displaying healthy adventitious roots 52 days after preparation. Leaf growth occurred prior to root growth (B) *Zamia integrifolia* stem cutting displaying healthy adventitious roots and simultaneous leaf growth 58 days after preparation. Credit: B. Deloso.

Results

Shortly after preparation of the stem cuttings in perlite, some leaflets on retained leaves abscised. The four treatments that included retained leaves did not reveal any differences in the extent of leaflet abscission. Regardless of treatment, 239 stem cuttings among the two studies generated adventitious root growth prior to resumed leaf growth, while some produced leaves and roots simultaneously (Figure 1).

The *Z. furfuracea* leaf retention study lasted 261 days from January 2018 to November 2018. The mean high temperature during this study was 35.5 ± 3.10°C, while the mean low temperature was 26.7 ± 1.39°C. The final cutting was assessed as dead on 01 November 2018, therefore the study was terminated on this date. The ANOVA revealed no significant effect of the experimental treatments on the days to first root contact, days to initial leaf emergence, root dry weight, stem dry weight, leaf dry weight, or number of coralloid clusters (Table 1). The percent rooting success was 92% for plants with Root-Drench, 100% for plants with Root-Drench + Wiltpruf, 88% for the plants with Wiltpruf, 96% for the control plants with leaves, and 96% for the control plants with no leaves. The overall percent rooting success of these *Z. furfuracea* cuttings was 96%.

The *Z. integrifolia* leaf retention study lasted 353 days from August 2018 to August 2019. The mean high temperature during this study was 34.3 ± 2.17°C while the mean low temperature was 25.9 ± 1.05°C. The final three unrooted stem cuttings were assessed as dead on 07 August 2019, therefore the study was terminated on this date. The ANOVA revealed no significant effect of the experimental treatments on the days to first root contact, days to initial leaf emergence, root dry weight, stem dry weight, leaf dry weight, or number of coralloid clusters (Table 2). The percent rooting success was 96% for the plants with Root-Drench, 100% for the plants with Root-Drench + Wiltpruf, 92% for the plants with Wiltpruf, 96% for the control plants with leaves, and 100% for the control plants with no leaves. Rooting success percentage was 96% for these *Z. integrifolia* cuttings.

The radial sections of the rooted stem cuttings revealed similar behavior for the two *Zamia* species. For the base of every cutting, the adventitious roots were initiated within the space occupied by the stem's vascular cylinder. The vascular tissues within the adventitious roots displayed integrity with the pre-existing vascular tissues in the stem cuttings.

Discussion

Leaf retention on stem cuttings has proven beneficial for propagation of many horticultural species (Hartmann et al., 1990), but our primary objective of determining how leaf retention affected AR formation on stem cuttings of two *Zamia* species revealed no beneficial or detrimental influence on propagation success or the speed of adventitious root formation. Our results indicated that the disparity among various anecdotal recommendations that are expressed in the cycad literature

Table 1. Traits of *Zamia furfuracea* Stem Cuttings Following Leaf Retention Treatments.

Response variable	Control no leaves	Control with leaves	Root-Drench	Wiltpruf	Both anti-transpirants	$F_{4,114}$	*P*
Time to first root contact (days)	56	62	58	61	67	0.032	0.23
Time to leaf emergence (days)	50	62	63	70	75	1.260	0.55
Coralloid root clusters (clusters)	9	8	12	12	8	2.305	0.06
Root dry weight (g)	6.94	9.56	11.07	9.75	9.21	1.965	0.10
Stem dry weight (g)	24.39	25.18	26.61	26.64	24.54	0.823	0.51
Leaf dry weight (g)	18.56	19.24	24.72	22.45	21.10	1.870	0.12

Root-drench anti-transpirant reportedly stimulates abscisic acid production in roots. Wiltpruf anti-transpirant forms coating over leaf surfaces. $N = 25$.

Table 2. Traits of *Zamia integrifolia* Stem Cuttings Following Leaf Retention Treatments.

Response variable	Control no leaves	Control with leaves	Root-Drench	Wiltpruf	Both anti-transpirants	$F_{4,114}$	P
Time to first root contact (days)	70	75	73	76	75	0.086	0.98
Time to leaf emergence (days)	67	74	79	77	81	1.010	0.40
Coralloid root clusters (clusters)	11	8	11	10	9	1.435	0.22
Root dry weight (g)	8.57	8.66	9.93	8.70	8.11	0.442	0.77
Stem dry weight (g)	14.25	12.36	15.22	13.54	12.88	1.062	0.37
Leaf dry weight (g)	8.57	10.73	11.96	11.06	10.40	1.397	0.23

Root-drench anti-transpirant reportedly stimulates abscisic acid production in roots. Wiltpruf anti-transpirant forms coating over leaf surfaces. $N = 25$.

concerning leaf retention on cycad cuttings (Jones, 1993; Tang, 1985; Whitelock, 2002) is justified and the decision to retain or remove leaves from cycad stem cuttings is best guided by the preferences of the practicing horticulturist.

The problem of water loss in stem cuttings with retained leaves may be mitigated by the use of anti-transpirants to reduce stomatal gas exchange, but our secondary objective of determining how anti-transpirants influenced behavior of leafy stem cuttings of two *Zamia* species revealed no influence on propagation success. Moreover, leafless cuttings required no support to remain stable in the perlite, but the leafy cuttings required the added expense of providing a physical support frame to keep them from lodging. The results indicated that the added costs associated with using leafy cuttings and purchasing these commercial products is not warranted for cycad propagation protocols.

Application of anti-transpirants prior to excavation of horticulture and silviculture transplants has been used to improve survival (Anderson & Kreith, 1978; Hummel, 1990; Marshall et al., 1991; Simpson, 1984; Williams et al., 1990). To our knowledge, anti-transpirants have not been used for improving transplantation of cycad plants. This may be due to the relative ease with which cycad plants can be transplanted. Application of anti-transpirants to excised leafy cuttings has also been used with several crops to improve AR formation or grafting success (Baggot & Joiner, 1978; Gur et al., 1986; Lyons et al,. 1985; Marler, 2018; Switras-Meyer & Gillman, 2002; Whitcomb et al., 1974). We have shown that two *Zamia* species do not benefit from the use of these products, but this established literature indicates that more research is warranted to determine if there may be an application in cycad horticulture where anti-transpirants are beneficial.

Successful AR formation on leafless stem cuttings is frequently achieved by most experienced cycad horticulturists. However, the negative experience of waiting more than one year for resumption of growth is also a common phenomenon (Dehgan, 1999; Marler & Cruz, 2017). Some of the *Zamia* stem cuttings used in our studies exhibited this behavior, in that they required up to eight months to exhibit root or new leaf growth. Even if AR formation in the leafy cuttings was not better than the leafless cuttings, we were expecting the additional photosynthesis of the retained leaves in the leafy cuttings to potentially hasten AR formation and shorten the nursery time. Some of the leaflets abscised in the weeks following preparation of the stem cuttings, however the rachis of many cycad plants is photosynthetic (Norstog & Nicholls, 1997). Unfortunately, none of the treatments with leaf retention were capable of reducing this undesirable behavior of lengthy delays in the resumption of growth in cycad stem cuttings.

Our findings are likely applicable for stem cuttings from other cycad genera, based on our collective experiences. Our results highlight the relative ease and high

Figure 2. *Zamia furfuracea* stem cutting after 8 months of growth. Credit: B. Deloso.

survivability that is achievable when propagating cycad plants asexually. Regardless of treatment, a mean of 96% of stem cuttings produced healthy adventitious root systems and leaves by the end of both studies (Figure 2). Successful asexual propagation of cycads is possible when hygienic conditions are adhered to, stem cuttings are obtained from healthy plants (Marler, 2018), a fungicide mixture such as flowers of sulfur and Bordeaux is applied to the cut surfaces (Tang, 1985), a sealant is used to cover cut surfaces to prevent desiccation (Marler et al., 2020), the medium demonstrates adequate aeration and drainage capacity, and underwatering is used during the propagation phase (Whitelock, 2002).

Our experimental approach to determining the influence of a horticultural treatment on asexual propagation is unprecedented. This is the first replicated study that directly addressed the issue of leaf retention on cycad stem cuttings. We used a robust number of replications that has been used in only one other study (Deloso et al., 2020), while the number of replications was not reported for most of the past reports on cycad horticulture studies. Moreover, most of the literature on cycad propagation is anecdotal and no data were reported to support the published recommendations. Our use of destructive sampling at the end of the studies to obtain dry weights for the purpose of quantifying plant growth has also been used in only one other study (Deloso et al., 2020).

In summary, leafless and leafy stem cuttings of two *Zamia* species served as control treatments and exhibited highly successful AR root formation of 96%. Using commercial products designed to reduce water loss from plant leaves did not influence the percentages or speed of propagation success for stem cuttings. In our previous research we detailed efforts by cycad nurseries in rural municipalities to aid in reintroduction efforts of cycads back to wild populations and supplement local farmer income (Deloso et al., 2020). Indeed, cycad conservationists in countries where these commercial products are difficult to procure need not go to the expense of trying to obtain these products. We believe that our results are applicable to other cycad species based on our collective experiences, but this remains to be studied. Future studies should include species from the other nine cycad genera to determine if they exhibit similar behavior. In addition to using a robust number of replications, other experimental groups could be added to future studies including: retaining all of the leaves on a stem cutting and testing other anti-transpirant products. Moreover, we did not closely examine the stomata on the leaves in our studies. Closer inspection of the stomata on cycad leaves in future studies would shed light on the efficacy of anti-transpirant products that reportedly increase ABA production and trigger stomata closure. Indeed, most of the 358 described cycad species (Calonje et al., 2020) have not been represented in the asexual propagation literature.

Implications for Conservation

Any improvement in the protocols for vegetative propagation of cycads by stem cuttings may be of crucial importance for conserving the most threatened species. This study adds to the call for more applied horticultural research on cycads to aid in their conservation efforts (Donaldson, 2003). There is a wealth of information in cycad lore on whether to retain leaves on cycad plants during propagation protocols, yet no previous cycad horticulture study has empirically examined whether retaining leaves has any benefit. Our results have helped to alleviate some of this ambiguity. Cycads are the most endangered group of plants globally and horticultural research on the group has been lacking despite calls for an increase in such research to aid in conservation of this remarkable group of plants (Donaldson, 2003).

Transplanting of cycads with leaves may have conservation applications when populations of cycads are destined for destruction and no other alternative is available except excavation. For example, *Encephalartos woodii* Sander is extinct in the wild and the only known multi-stemmed male plant was excavated from the Ngoye Forest, KwaZulu Natal between 1903 and 1916

(Osborne & Paschke, 1993). The main stems were transplanted to Durban botanical gardens in South Africa where they are still living to this day (Osborne, 1986). Cycads are dioecious and *E. woodii* is only known from a single male plant, therefore this species is dependent on asexual propagation protocols for its continued survival. Several other African cycad taxa are extinct in the wild and many other cycad populations worldwide are unfortunately on a downward trend (Bamigboye et al., 2016). Removal of plants in the wild for *ex situ* conservation may be the only viable option to save species that are trending towards extinction in the wild, and the use of anti-transpirants may aid in the successful transplanting of large specimens.

One of the principal threats to *in situ* cycad populations worldwide is illegal collection of plants in the wild for the horticulture trade. One of the species we used in these studies, *Z. furfuracea*, is one such cycad species that has suffered historically from this threat (Chemnick & Gregory, 2010). Our study and similar studies may help to inform how the horticulture nursery industry can meet the horticultural demands in an effort to stop wild-harvesting of plants. In summation, cycads have been understudied in the horticultural sciences and future horticultural research into the group will greatly benefit their conservation.

Acknowledgments

We thank Frankie Matanane for assistance with nursery maintenance and help with preparation of the stem cuttings. We thank Constance Sartor for image editing and preparation.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was funded by United States Forest Service grant number #13-DG-11052021-210.

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