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Research Article

Plastic responses mediated by identity recognition in below-ground competition in *Cycas micronesica* K.D. Hill

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Abstract
We studied the ability of *Cycas micronesica* roots to recognize the identity of competitor roots using pot culture. Pairs of seedlings were grown in single pots for 10.5 months, and morphological traits of the focal plant were determined when the non-focal plant was a half-sibling, a close relative from a mother 170 m away, a distant relative from a habitat 40 km away, or a different species (*Cycas edentata*) from Mindanao, Philippines. Most of the measured response traits exhibited similar trends and confirmed that *C. micronesica* plant roots were able to recognize the identity of competitors. For example, focal plants grown in pots with *C. edentata* competitors exhibited 66% increase in fine root dry weight, 54% increase in coralloid root dry weight, and 49% increase in total plant dry weight, compared to focal plants grown in competition with half-siblings. Our methods have shown that plot design in *ex situ* conservation and botanic garden settings would improve by positioning *C. micronesica* plants adjacent to non-relatives. Recruitment may improve within *in situ* conservation plots if managers distribute seeds away from the maternal parent so that emerging seedlings are not forced into immediate competition with half-siblings. These findings may be useful for designing more complex experiments to quantify the influence of these plastic root responses on plant nutrient status and physiology.

Keywords: cycad, *Cycas edentata,* kin selection, phenotypic plasticity

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Introduction

The roots of many plant species are able to distinguish the identity of roots of competing plants. Differences in root response to self roots, roots of a close relative, or roots of non-relatives have been reported [1-5]. When the plastic response reduces investment into competitive behaviors, the fitness of two competing plants may increase as a result of the cooperation [6].

This plant root behavior was recently reported for the gymnosperm *Cycas edentata* de Laub. [7], which was the first known report of identity recognition traits of plant roots for a gymnosperm [8]. The results indicated that kin recognition abilities by plant roots may be an ancient plant trait. *Cycas edentata* is a widespread species found in coastal zones of many oceanic islands [9]. *Cycas micronesica* exhibits similar population traits, with an indigenous range that includes islands in Palau (local name remiang), Yap (local name faltir), Guam (Fig. 1), and the Northern Mariana Islands (local name fadang) [10]. This once abundant arborescent cycad species has become threatened by the 2003 invasion of the armored scale *Aulacaspis yasumatsui* Takagi into its native range [11]. The widespread plant mortality generated an Endangered listing in 2006 by the International Union for Conservation of Nature and Natural Resources [12], and the species received United States Federal protection when it was listed as Threatened in 2015 [13].

A robust off-site conservation planting of Guam's population of *C. micronesica* was established in an isolated locality of Tinian island in 2007 [14]. Growth of individuals within this minimally managed germplasm collection has been highly heterogeneous (Fig. 2). Conservation of the germplasm from some Guam habitats has been compromised by this phenomenon, and experimental and observational approaches to determine possible causal mechanisms are needed to continue effective management practices.

Our objectives herein were to determine if *C. micronesica* roots were able to recognize the identity of a competitor, and if altruistic behaviors would mediate the plastic responses of *C. micronesica* plants that were grown with root competition. The results may improve management decisions for *ex situ* conservation plantings of the species by informing decisions about positioning plants by genotype and for *in situ* conservation programs by improving knowledge about kin cooperation.

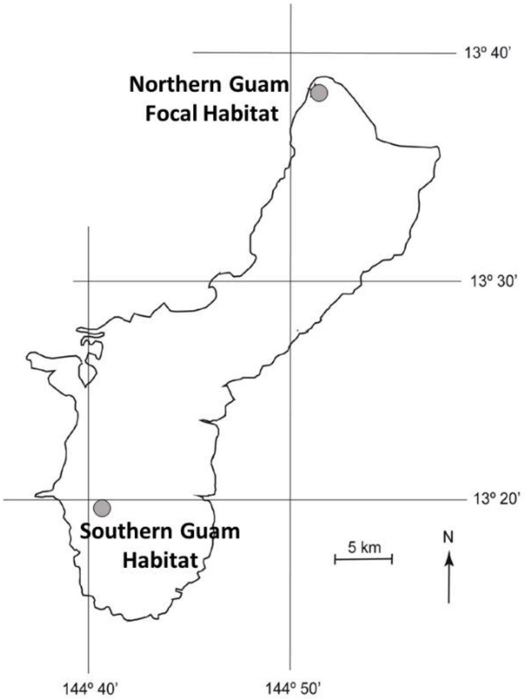

Fig. 1. Map of United States Territory of Guam depicting focal *Cycas micronesica* habitat in the north where seeds for half-sibling competitors and nearby maternal parent competitors were harvested, and habitat in the south where seeds for distant habitat competitors were harvested.

Fig. 2. Six-year-old Guam *Cycas micronesica* plants growing in off-site germplasm collection in Tinian Island, revealing example of plants that exhibit minimal growth rates but otherwise appear healthy (left), and example of plants that exhibit robust growth rates (right). (Photo credits: Thomas Marler)

Methods

Mature *C. micronesica* seeds were harvested from a single female tree in southern Guam on 12 October 2013, and from two female trees in northern Guam on 14 October 2013 (Fig. 1). One of the two trees in northern Guam was designated as the focal plant. A distance of 170 m separated the two trees in northern Guam, and a distance of 40 km separated the focal plant from the southern Guam habitat. Mature *C. edentata* seeds were purchased from the owner of a copra farm, and were harvested from a single female tree in north-east Mindanao Island, Philippines on 25 October 2013. The sarcotesta was cleaned from all seeds, which were then stored in ambient conditions for two months.

The seeds were placed under germination conditions beginning 1 January 2014 by placing them on top of a moist perlite bed and providing 100% humidity within closed plastic containers. Under these conditions, seeds with mature embryos began germination by growth of the coleorhiza through the hard sclerotesta tissue. These germinated seeds were removed from the perlite on 2 February 2014 and planted in the 2.6-L experimental containers.

The experiment was conducted at the University of Guam research nursery in Angeles City, Philippines. The container medium was composed of nine parts washed river sand quarried from the Pasig-Potrero River and one part topsoil from secondary forest in Barangay Haduan, Angeles City, Pampanga. This medium had a pH of 6.1, and nutrient status was limited (Table 1). One seed from the *C. micronesica* focal plant served as the experimental unit for each treatment, and one seed from one of four treatments was planted in the same container to form seedling pairs. The four competition treatments were: (1) a half-sibling from the same maternal parent, (2) a nearby relative from the maternal parent 170 m away, (3) a distant relative from the maternal parent 40 km away, and (4) a *C. edentata* competitor.

Table 1. Chemical elements ($\mu g \cdot g^{-1}$) of container substrate in *Cycas* seedling competition study.

Substrate property	Value
Total carbon	4890 ± 1100
Total nitrogen	310 ± 113
Available phosphorus	96.7 ± 3.9
Extractable potassium	50.7 ± 4.3
Extractable calcium	424 ± 13
Extractable magnesium	40.3 ± 4.3
Extractable manganese	17.3 ± 1.9
Extractable iron	8.0 ± 1.5
Extractable copper	1.0 ± 0.1
Extractable zinc	9.8 ± 1.5

Mean ± SE, n = 3.

The containers were placed on a raised wire mesh bench 1.22 m in width. Each container was provided with 625 cm^2 of bench space, and a border row of containers surrounded the experimental plants. General growing conditions of this nursery were previously described [7]. Briefly, the nursery bench received about 4 h of direct sun exposure in the morning, then filtered shade from a *Sandoricum koetjape* Merr. tree in the afternoon. The plants were irrigated daily to minimize competition for water resources.

A germinated *Cycas* seed spends the first few weeks to months of its life developing a robust taproot (see Fig. 1 in [7]). Therefore, the timing of ultimate leaf emergence to begin above-ground growth is highly variable for seeds that germinate simultaneously. We culled containers that took more than 4 weeks between leaf emergence dates for the two seeds, because the first seedling to grow would preempt the competition for light resources and benefit from new photosynthates for growth at an earlier date than the subsequent seedling. These competitive advantages of the initial seedling may have masked responses to our root competition treatments. We started with 13 containers per treatment but ultimately included six replications that exhibited homogeneous leaf emergence dates for all seedlings. The remainder of the containers were used as the border plants on the bench.

The plants remained in competition until ending measurements were conducted 16-22 December 2014. Final measurements and methods of analyses generally followed those of Marler [7]. The growing medium was gently washed from the co-mingled root systems for each pair of seedlings, then the seedling pair was photographed (Fig. 3). The roots were gently disentangled to harvest the northern Guam *C. micronesica* focal plant from each replication. Leaf and leaflet number per leaf were counted, then stem height and diameter were measured. The above-ground plant components were separated into leaflet, petiole+rachis, and stem sections. The root system was then separated into taproot, fine roots, and coralloid roots. Taproot length was measured directly, and fine root length was determined by the line intersect method [15,16]. The six separated components of each replication were dried at 75°C in a forced-draft oven, then dry weight was measured.

Several derived response variables were calculated. The sum of dry weight for the six components of each plant determined total plant dry weight. The shoot/root quotient was calculated by dividing dry weight of the three root components into dry weight of the three above-ground components. Specific root length of fine roots was determined by the quotient length/dry weight.

Each response variable was subjected to one way analysis of variance. Means separation for the models that were significant was conducted by least significant difference.

Results

The root system of *C. micronesica* plants grown in competition with *C. edentata* plants accumulated 72% greater fine root biomass and 66% greater fine root length than plants grown in competition with half-siblings (Table 2). The response was intermediate for plants grown in competition with close relatives and distant relatives. The taproot of focal plants in competition with *C. edentata* plants had 32% more biomass than that of plants in competition with half-siblings, but taproot length was unaffected by the competition treatments. Coralloid root biomass of focal plants grown in competition with *C. edentata* plants was 54% greater than that of plants grown in competition with half-siblings. Coralloid root biomass was not different among the three treatments that employed a competing *C. micronesica* plant. Specific root length was not influenced by genotype of the competitor plant.

Table 2. Response variables of focal *Cycas micronesica* plants from northern Guam habitat that were grown with half-sibling competitors or competitors from a nearby maternal parent, competitors from a parent in a distant habitat from southern Guam, or competitors from a *Cycas edentata* parent from Mindanao, Philippines.

Response variable	Half-sibling	Same habitat, different mother	Different habitat, same island	Different island, different species	Significance
Fine root dry wt (g)	1.76a	1.95ab	2.22b	3.02c	0.0002
Fine root length (cm)	701a	739a	878a	1165b	0.0053
Taproot dry wt (g)	2.75a	3.17b	4.18c	3.63c	0.0108
Taproot length (cm)	193	235	242	223	0.1717
Coralloid root dry wt (g)	0.57a	0.54a	0.56a	0.88b	0.0030
Specific root length ($cm{\cdot}g^{-1}$)	391	373	396	397	0.9503
Stem ht (mm)	54a	56a	59ab	63b	0.0500
Stem dia (mm)	32a	34ab	36bc	40c	0.0004
Stem dry wt (g)	7.00	7.92	9.63	8.44	0.1948
Leaves per plant	2.80	3.00	3.2	3.7	0.1934
Leaflets per leaf	15a	15a	16a	19b	0.0001
Leaflet dry wt (g)	5.05a	5.21a	6.72b	8.13c	0.0006
Rachis+petiole dry wt (g)	3.10a	3.57b	3.58b	5.91b	0.0001
Shoot dry wt / root dry wt	3.01	2.97	2.90	3.02	0.9394

Any two means within a row not followed by the same letter are significantly different based on least significant difference. N = 6.

Leaf and stem responses to the treatments were similar to those of the root system. The stems of focal plants grown in competition with *C. edentata* plants had 17% greater height and 25% greater diameter than stems of plants grown in competition with a half-sibling plant (Table 2). Stem dry weight was not influenced by the competition treatments. The number of leaflets per leaf was 27% greater for focal plants grown in competition with *C. edentata* plants than for plants grown in competition with half-siblings. This leaf trait was not different among the three treatments with *C. micronesica* competitors. Leaf number per plant was not influenced by competitor genotype. The leaflet component of leaves of focal plants grown with a *C. edentata* competitor was 61% greater in dry weight and the structural component of these leaves was 91% greater in dry weight than those of plants grown with a half-sibling competitor.

The response traits were integrated at the whole plant level such that total plant dry weight exhibited trends that were similar to the trends of the individual plant components (Fig. 4). The total dry weight of focal plants grown in competition with *C. edentata* plants was 49% greater than that of plants grown in competition with half-siblings. The plants grown in competition with a close relative or a distant relative were intermediate in biomass accumulation. Biomass partitioning into above-ground components in relation to below-ground components was not influenced by competitor genotype (Table 2).

Fig. 3. General appearance of healthy *Cycas micronesica* and *Cycas edentata* seedlings that were bare-rooted after being grown with root competition from February to December 2014. White arrow points to coralloid root clusters within which cyanobacterial endosymbionts fix nitrogen. (Photo credit: Thomas Marler)

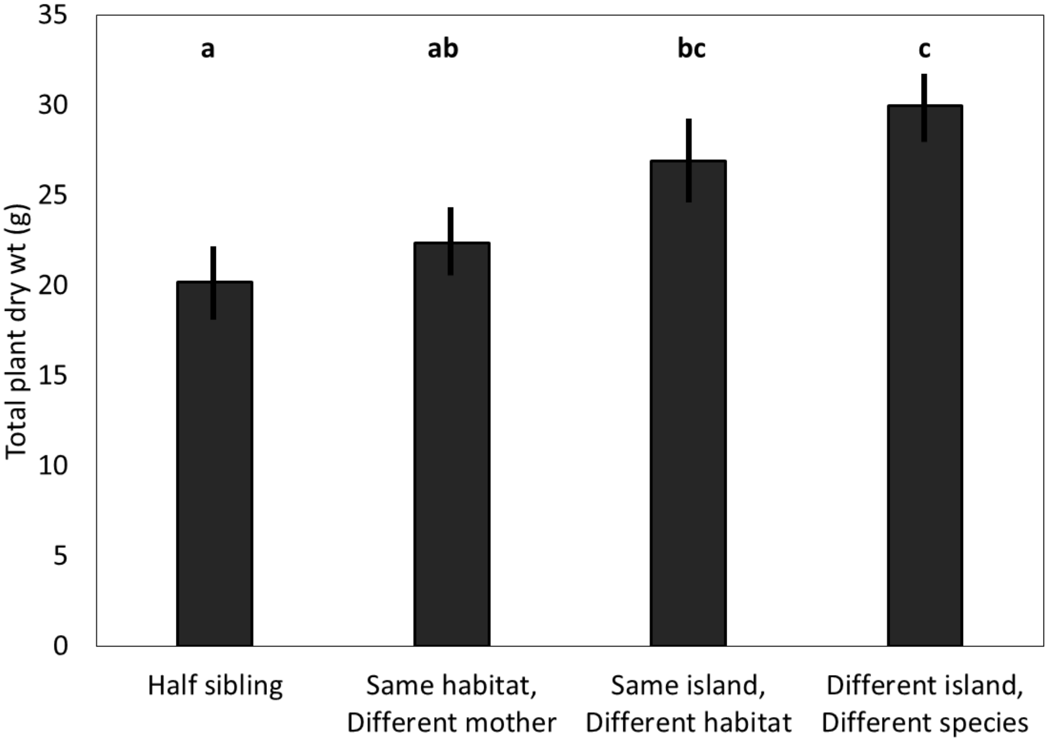

Fig. 4. Total plant dry weight accumulation by northern Guam *Cycas micronesica* plants that were grown with half-sibling competitors or competitors from a nearby maternal parent, a parent from a distant habitat in southern Guam, or from a *Cycas edentata* parent from Mindanao, Philippines. Mean ± standard error, n=6. Bars sharing same letter are not significantly different, P<0.0001.

Discussion

We have demonstrated identity recognition abilities for *C. micronesica* roots using shared rooting volume in pot culture. This is the second cycad species confirmed to possess these traits, as split-root techniques were used to demonstrate plastic responses of *C. edentata* roots to competitor identity [7]. In both studies, the greatest growth increases occurred for focal plants growing in contact with a different species. Increases in fine root growth were substantial for plants competing with non-relatives, and for both species the increases occurred strictly through greater biomass in the lateral root system, since specific root length was not influenced by competitor identity.

One consequential difference between the *C. edentata* response and the *C. micronesica* response was coralloid root growth. Competitor identity did not affect coralloid root biomass for *C. edentata* plants [7], but coralloid root biomass was 54% greater in *C. micronesica* plants competing with congenerics than in plants competing with half-siblings (Table 2). The manipulations required to construct the split-root experimental units may have muted this root response for the *C. edentata* study. Alternatively, the disparate responses may be an example of inter-specific differences in kin selection traits.

Maximizing root length is critical when plants compete for rhizosphere nutrients with neighbors [17,18]. Therefore, any conservation management action that generates an increase in root length has the potential to improve plant growth and health. Additionally, although nothing is known about how the mineral elements of cycad plants are influenced by the extent of coralloid root development, this specialized cycad root structure carries an analogous function in some respects to the root nodules in a legume. Recent work with *Vigna unguiculata* L. Walp. [19] indicated that as root nodule number and weight increased, the expected increase in *Rhizobia*-fixed nitrogen was associated with a concomitant increase in a majority of other rhizosphere-derived essential mineral elements in the leaf tissue. If this strong relationship between endosymbiont nitrogen-fixation and general mineral accumulation in legumes is similar in cycads, the implications of our results are of critical importance to conservation managers.

Specific root length for fine roots averaged 894 $\mathrm{cm \cdot g^{-1}}$ in the *C. edentata* plants [7], and 389 $\mathrm{cm \cdot g^{-1}}$ for *C. micronesica* plants. While the thinner roots in the *C. edentata* study may have been a response to the split-root manipulations, an alternative explanation is that inter-specific variation in fine root morphology and foraging traits may be substantial in this genus.

More complex experimental approaches would help to confirm some of these findings. Soil cores obtained from *ex situ* plantings of various cycad species would be a simple way to quantify heterogeneity in root traits among species within a relatively homogeneous setting. Additionally, experimental manipulations that influence coralloid and fine root growth may be useful for designing studies to quantify the influence of these plastic root responses on tissue nutrient status and whole plant physiology.

Implications for conservation

Our research shows how conservation decision-makers and managers may be able to improve plant growth by exploiting beneficial root traits exhibited by *Cycas micronesica* plants when they are grown adjacent to non-relatives. Stronger intraspecific than interspecific competition during the seedling stage has been reported for another cycad species, *Dioon sonorense* growing in arid conditions [20]. Reduced root growth due to kin cooperation may explain part of the increased intraspecific competition reported for this other cycad species, suggesting that our findings may be widespread among cycad species. The most relevant application of our methods is in conservation nurseries, where conditions of seed germination and early seedling growth are strongly influenced by management decisions. Managers may need to reconsider traditional approaches of germinating batches of sibling seeds in community containers, as these protocols force germinating seedlings into contact with close relatives, and the altruistic responses may reduce plant growth.

The results may also be used in *ex situ C. micronesica* germplasm collections, such as the germplasm that is being conserved in Tinian Island [14]. A plot layout that integrates germplasm into heterogeneous plantings where close relatives are not positioned in adjacent planting sites may allow a conservation manager to improve overall plant nutrition through increased coralloid root development and increased fine root deployment. *Cycas micronesica* is also being conserved in fenced *in situ* management plots in north Guam. In these plots, recruitment potential may be improved if managers collect and then distribute seeds within the plots to locations that are distant from the maternal parent. These isolated germinating seedlings could escape the immediate competition with half-siblings that would occur if they germinated directly beneath the maternal parent where they naturally fall.

One caveat in applying this knowledge to field sites is that “biological space” available to each plant may modify the root behaviors that we documented within the containers. Indeed, physical underground space may be considered a distinct resource [21,22] and confirming the relative value of using heterogeneous genotypes in adjacent planting sites to increase beneficial competitive behaviors will require more field research.

Nascent conservation programs for rare plant species are often initiated with very little species-specific research. The *C. micronesica ex situ* conservation plots in Tinian and *in situ* conservation plots in northern Guam are local models for other rare Western Pacific plants. When conservation programs are initiated in the absence of an established literature or knowledge base, adaptive management requires continual research based on local observations and empirical evidence. Our results provide new insights for decisions that will improve ongoing conservation actions for *C. micronesica*.

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