Predicting the risk of non-target damage to a close relative of a target weed using sequential no-choice tests, paired-choice tests and olfactory discrimination experiments

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Abstract

We investigated host plant utilisation by the candidate biocontrol agent Paradibolia coerulea (Coleoptera: Chrysomelidae) on the target plant Spathodea campanulata Beauv. (Bignoniaceae) and a closely related non-target plant, Kigelia africana (Lam.) Benth. (Bignoniaceae). Paired-choice and sequential no-choice experiments were performed and coupled with olfactory discrimination experiments to test the insects' responses to volatiles from both plant species as well as to cues from conspecific beetles. Although K. africana was utilised by P. coerulea, S. campanulata was preferred for both adult feeding and oviposition. Interestingly, whereas females were attracted to olfactory cues emitted by S. campanulata, males demonstrated no such olfactory discrimination. Females were also attracted to cues deposited by males, and males were deterred by cues from other males, but neither sex~~es~~ responded to female olfactory cues. Very few eggs were recorded on K. africana and none of the larvae that hatched on K. africana survived the first instar. Both S. campanulata and K. africana are suitable for adult feeding, but persistent utilisation of K. africana in the field is unlikely because larval development is only possible on S. campanulata and because the adult females are strongly attracted to volatiles emitted by the target plant.

Nevertheless, if P. coerulea is released as a biocontrol agent, spill-over adult feeding could potentially occur on K. africana growing sympatrically with S. campanulata. Because P. coerulea cannot complete its development on K. africana, non-target damage will only occur where the target plant is present, with an intensity dependent on densities of adult beetles locally.

Keywords: Paradibolia coerulea; Spathodea campanulata; Kigelia africana; weed biological control; host specificity testing; host plant selection

1. INTRODUCTION

Spathodea campanulata Beauv. (Bignoniaceae) (African tulip tree) is a problematic alien invasive species in many tropical and subtropical parts of the world (Larrue et al., 2014). It is native to Central and West Africa (Bidgood, 1994), but widespread cultivation has led to naturalization in areas outside of the native range (Francis, 1990). It is considered one of the most damaging invasive alien species in the world due to the negative impact of infestations on native biodiversity and agricultural productivity (Lowe et al., 2000; Labrada & Medina, 2009; Larrue et al., 2014). Spathodea campanulata is considered a suitable target for biological control in the Pacific region (Paynter, 2010) and biological control options are being investigated (Paterson et al., In Press). One of the potential candidate biological control agents is a flea-beetle, Paradibolia coerulea Bryant (Chrysomelidae: Coleoptera), which feeds externally on the leaves of the plant as an adult and mines between the epidermal layers of leaves as a larva (Paterson et al., In Press).

Spathodea campanulata belongs to the family Bignoniaceae, which contains many species of ornamental and cultural importance (Olmstead et al., 2009). It is part of the

paleotropical clade of Bignoniaceae which has an African and Asian distribution (Olmstead et al., 2009). The closest relatives to the target weed are considered to be other African Bignoniaceae species including the African sausage tree, Kigelia africana (Lam.) Benth. (Olmstead et al., 2009). Although K. africana is not indigenous to the Pacific region, where S. campanulata is most problematic, it is present as a horticultural plant in some parts of the region. K. africana is also indigenous to South Africa, where S. campanulata has been declared an invasive alien plant (Department of Environmental Affairs, 2014).

Spathodea campanulata is a large tree of up to about 30m in height, has compound leaves with four to eight pairs of opposite leaflets and a terminal leaflet. Each leaflet is approximately 10cm long and 5cm in width. The flowers are large, bright red and bell-shaped; and the fruits are cigar-shaped woody pods which contain winged seeds (Hedberg et al., 2006). Kigelia africana is also a large tree, reaching 25m in height with compound leaves containing six to ten leaflets of similar dimensions to those of S. campanulata (Coates-Palgrave, 2002). The flowers of K. africana are also similar to those of S. campanulata, but are a deeper red and are found on long flexible stems that hang down from the mature woody branches. Kigelia africana has distinctive large sausage-shaped woody fruits of up to 1m in length and weighing up to 10kg (Coates-Palgrave, 2002).

Close relatives of target weeds are the most likely species to be fed on as alternative host plants by candidate control agents and it is now standard practice to select test plants on the basis of phylogenetic separation from the target weed, starting with the closest relative and incorporating more distantly-related taxa until the host-range is circumscribed (Briese, 2005). Host range has almost exclusively been determined by employing test designs that incorporate (i) no-choice, (ii) choice and

(iii) open-field (multi-choice) trials (Schaffner, 2001; Moffat et al., 2013). The no-choice test applies a single biological control agent with a single test plant species under standard conditions (Withers & Mansfield, 2005). Over an extended experimental duration, no-choice tests may overestimate the fundamental host range of a candidate agent, because over time there is increased acceptance of hosts due to starvation and experience (Withers, Barton-Browne & Stanley, 1999). Choice tests, in which the potential agent is exposed to two or more test species that include the target plant, provide an indication of preference by the insect (Buckingham, Okrah & Christian-Meier, 1991; Edwards, 1999). In order to interpret the results of choice tests it is important that the mechanism of host plant selection be considered. For example, an insect may be stimulated to oviposit on a test plant that it would never utilise under field conditions if plant volatile cues from the target weed have accumulated around the non-target test plant. Multi-choice tests under open field conditions are therefore a more reliable method of determining the realised host range of potential agents, but these tests are not always possible, because agents must usually be kept under quarantine conditions. Under field conditions, host plant selection will be determined by a number of cues that may not be present, or may be distorted, under the artificial conditions simulated in quarantine, so an understanding of how a potential agent uses cues to select a host plant should improve interpretation of the results of host specificity testing (Marohasy, 1998).

The sequential no-choice test is an infrequently-utilised test design which may reduce the probability of obtaining false (positive or negative) results compared with standard host specificity tests (Withers, Barton-Browne & Stanley, 2000). This test design effectively combines several no-choice tests into a single experiment, whereby the candidate biological control agent is offered a series of test plant species,

101 alternating between the target species and an additional test species (Fig. 1). The
102 value of the sequential no-choice test is that volatiles emitted by the target plant are
103 less likely to confound the utilization of other test plants, that behavioural aspects of
104 host-selection (such as learning and experience) are incorporated into the test design
105 by exposing the herbivore to test plants on multiple occasions, and because time-
106 dependent influences on host-acceptance and utilization are accounted for (Withers,
107 Barton-Browne & Stanley, 2000).

108 Host plant selection is a vital element in the survival of most herbivorous insects,
109 but species with less mobile immature stages, such as endophagous beetle larvae,
110 are particularly reliant on appropriate decisions being made by ovipositing females
111 that select plants offering suitable requirements for larval development (Bernardo,
112 1996; Singer, 1986; Casagrande & Dacey, 2007). The agent screening process
113 utilised in biological control programs could be significantly improved if the behavioural
114 and physiological mechanisms which mediate host selection and acceptance are
115 better understood (Marohasy, 1998). A range of sensory cues are utilized by insects
116 to locate their hosts, including: visual (Fischer et al., 2004), olfactory (Visser, 1986)
117 and tactile/contact cues (Müller & Hilker, 2001). The efficiency and reliability of each
118 cue can be variable, but Heisswolf et al. (2007) proposed that olfactory cues are more
119 reliable than both visual and many contact cues for chrysomelid beetles (Bernays &
120 Chapman, 1994; Horiuchi et al., 2003).

121 Conspecific pheromones, or other aggregation cues, may also be involved in
122 host selection by certain chrysomelid beetles (Wood, 1982). Conspecific cues are not
123 usually explicitly considered in host specificity testing procedures, but in some
124 circumstances they could lead to false positive or false negative results. For example,
125 cues from one sex may influence host choice or feeding behaviour of the other sex,

126 leading to different results when both sexes are present or if one sex is tested alone.
127 A better understanding of the role of conspecific cues in host plant selection and
128 feeding behaviour could therefore help improve the reliability of host specificity testing
129 procedures.

130 In this study, data from sequential no-choice tests and paired choice tests were
131 combined with data from olfactory discrimination experiments in order to predict the
132 risk of non-target impacts to K. africana if P. coerulea were released as a biological
133 control agent in areas where both species are present.

134

135 **2. METHODS AND MATERIALS**

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137 2.1 Insect and Plant Cultures

138 Potted S. campanulata and K. africana plants were obtained from local nurseries and
139 housed in a greenhouse before being taken into quarantine for experiments.
140 Paradibolia coerulea individuals were collected from 10 natural populations in March
141 2014 in Ghana, West Africa. The 10 wild populations were combined into a single
142 culture population after importation into quarantine in South Africa. The P. coerulea
143 culture was maintained under a 16:8 hour light/dark regime and temperatures were
144 maintained at 25 ± 2 ℃ (S.E.). The culture was provided with potted S. campa nulata
145 when required. Larvae were transferred to 2 L tubs layered with potting soil, and
146 provided with fresh plant material daily. The soil was kept moist at all times to provide
147 a suitable pupation substrate.

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2.2 Sequential No-Choice Tests

Recently-eclosed naïve P. coerulea adults (eclosed within 24 hours of the experiment and not exposed to any food plant) were inoculated onto potted S. campanulata (test plant A) or K. africana (test plant B) plants, in an ABA and BAB sequence (Fig. 1). The plants were approximately 1m in height and were housed individually in standard 1.2m x 0.6m x 0.6m insect cages. Each cage was inoculated with a pair of adult beetles (1 female, 1 male). Beetles are readily sexed as female P. coerulea possess a red, vertical stripe on the dorsal surface of the abdomen, which is absent in male beetles. After 14 days, the plant~~s~~ species in each cage was alternated and the original P. coerulea pair was offered the alternate plant in the test sequence. After an additional 14 days, the beetle pair were offered again the test plant species provided during the first 14 days. A new, undamaged plant was used each time the plant was changed. Controls employed an AAA test sequence. The number of feeding scars, eggs, larvae, larval tunnels and leaves damaged on the plants were recorded for each of the plants in the sequence. The experiment was replicated five times.

2.3 Paired Choice Tests

Two recently-eclosed naïve P. coerulea adults (1 male, 1 female) were placed in insect cages (1.2m x 0.6m x 0.6m) with similar sized potted S. campanulata and K. africana plants. After 28 days the number of feeding scars, eggs, larvae, larval tunnels and leaves damaged were recorded. If the adults died during the 28 days then that replicate was discarded. The experiment was replicated six times.

2.4 Host Plant Olfactory Cues

The response of P. coerulea to olfactory stimuli from the two test plant species was determined using a stem-arena design (~~Muller~~ Müller & Hilker, 2000). Stems (approximately 60mm in length x 20mm in diameter) were erected by enclosing detached plant material within a piece of filter-paper that was shaped into a cylinder, to ensure that P. coerulea had no contact with, or visual of, plant material (e.g., Heisswolf et al., 2007). A petri dish (90mm x 12mm) was half-filled with soil ~~so that leaves wrapped in filter paper~~ and stems were ~~could be~~ pressed into the soil, allowing them to stand upright. The petri dish was placed in a 2 L tub, and covered with a fine mesh cloth to prevent any beetles from escaping the arena (see Heisswolf et al., 2007 for a graphical depiction of the stem-arena design). A single leaf (approximately 60mm x 40mm) of each test plant was placed within ~~rolled filter paper~~ a stem, and placed in the stem-arena. Controls were erected by placing a toothpick inside a ~~the rolled filter pap~~stem~~er~~. There were four stems used per arena, with two stems used per treatment, and the position of each treatment was randomly allocated to account for spatial bias. The treatments that were applied include: (i) Spathodea campanulata/Control, (ii) Spathodea campanulata/Kigelia africana and (iii) Kigelia africana/Control

The experiment was performed by introducing a single, naïve P. coerulea adult into the middle of the arena, allowing for a 5 minute acclimatisation period. Each beetle was sexed before being placed in the arena. The beetle was then observed for 30 minutes, recording the amount of time spent on each stem. Each treatment was replicated ≥ 9 times, using a beetle only once to control for the effects of experience and learning. Only beetles that made a choice within the allotted time were included in the analysis.

2.5 Conspecific Olfactory Cues

An aggregation test was employed to determine whether conspecific contact cues were involved in beetle location behaviour (Tansey et al., 2005). A single adult P. coerulea, between 12 and 15 days old, was sexed and then placed inside a glass vial (60 ~~c~~mm x 1~~.~~5 m~~c~~m) lined with filter paper. The beetle was left for 24 hours before being removed from the vial to allow for adequate deposition of any potential contact cues. Another P. coerulea adult was then introduced into the treated vial, which was connected to a second glass vial lined with untreated filter paper, and the two were sealed together with masking tape. The newly-introduced beetle was allowed to acclimate inside the apparatus for 30 minutes. The position of the beetle in the vials containing treated ("1") or untreated ("0") filter paper was scored as a binary response at 15 minute intervals over a two-hour observation period, whereby each two-hour observational period constituted a single replicate.~~.~~

Thirty replicates were performed for each treatment, which included: (1) female-male (female response to male contact cues), (2) male-female, (3) female-female and (4) male-male combinations.

2.6 Statistical Analyses

All data were subjected to normality testing by visually observing box plots and Shapiro-Wilks tests, and homogeneity of variances were analysed by performing Bartlett's Test. Data that failed to meet these assumptions were subjected to appropriate transformations, or analysed using non-parametric statistical methods.

Herbivore performance between test-plant species for paired-choice experiments was analysed by paired t-tests (Horton, 1995).

Sequential no-choice experiments were analysed by two-way analysis of variance (ANOVA) with regards to plant-sequence effect on the number of feeding scars observed. Paired t-tests were performed on contrasting treatments to determine whether prior experience on a test-plant influenced host-use. Reproductive output of P. coerulea was not subjected to statistical analyses as only P. coerulea pairs on S. campanulata produced larvae.

The preference of P. coerulea to stems (i.e., filter paper cylinders containing leaf material) of either test-plant or controls were analysed by binomial tests, which were further partitioned into male and female responses, to determine whether gender influenced olfactory host-discrimination. The latency period (i.e., time taken before a stem was chosen), (Heisswolf et al., 2007), was analysed by one-way ANOVA, and again was partitioned into gender-wise categories.

The responses obtained during the conspecific cues experiment were recorded as the number of times out of the possible eight sampling events that the beetles were observed in the vial containing the treated filter paper (denoted N_T). Kruskal-Wallis one-way ANOVA was used to determine any treatment effects. Post-hoc analysis was performed by implementing a multiple comparisons test after Kruskal-Wallis to elucidate which gender-combination treatments yielded significant responses. One-sample t-tests were performed to demonstrate whether the N_T responses differed from the hypothesized mean of four responses for each filter paper treatment (Tansey et al., 2005).

3. RESULTS

3.1 Sequential no-choice tests

Paradibolia coerulea demonstrated a clear preference for S. campanulata over K. africana in sequential no-choice experiments (Fig. 2). The test plant species offered to the beetle significantly influenced the number of feeding scars measured ($F_{1,20}$ = 348.56, P = <0.0001) (Fig. 2a), while a significant interaction between the sequence of plants offered and the test species was also observed ($F_{1,20}$ = 9.01, P = 0.0085).

~~P. coerulea~~ Beetle larvae were only observed on S. campanulata, with the majority of larvae found on the control plant sequence AAA (Fig. 2b). Larvae were not found for BAB sequence S. campanulata plants, however larvae were produced on the second presentation of S. campanulata during ABA sequence replicates, indicating a host-plant and temporal effect on the beetle's reproductive output (Fig. 2b).

3.2 Paired-choice tests

Paradibolia coerulea adults demonstrated a clear preference for S. campanulata during choice-test trials when offered both plant species together (Table 1). The number of feeding scars measured was approximately four times greater on S. campanulata than on K. africana ($t_{(5)}$ = 4.48, P = 0.0065) while the number of K. africana leaves damaged by adult feeding was less than half than the number of leaves damaged on S. campanulata plants ($t_{(5)}$ = 4.73, P = 0.0072). The number of eggs laid on S. campanulata was six times more than for K. africana ($t_{(5)}$ = 6.74, P = 0.0011), while the number of larvae observed on K. africana was approximately 20 times fewer than larvae on S. campanulata ($t_{(5)}$ = 5.71, P = 0.0023). The number of feeding tunnels was significantly greater on S. campanulata than K. africana ($t_{(5)}$ = 6.94, P = 0.0001),

274 with 28.17 ± 4.36 (mean ± S.E.) feeding tunnels on S. campanulata and only 2.00 ±
275 1.12 tunnels on K. africana. Only two larvae were observed on K. africana and neither
276 survived through to the end of the first instar (100 ± 0 % mortality), while larval mortality
277 on S. campanulata was significantly lower at 15 ± 6 % ($t_{(5)} = 17.12$, P = 0.0034).
278
279 3.3 Host Plant Olfactory Cues
280
281 The beetles demonstrated a clear preference for arena-stems containing S.
282 campanulata leaf material rather than empty controls (Fig. 3A, n = 10, P = 0.0107).
283 There was an appreciable attraction to K. africana leaf material rather than to the
284 controls although this result was not significant (Fig. 3B, n = 13, P = 0.1334).
285 Paradibolia coerulea demonstrated a preference for S. campanulata leaf material
286 over the K. africana leaves (Fig. 3C, n = 29, P = 0.0121). Female beetles demonstrated
287 a clear preference for S. campanulata (Fig. 3D, n = 20, P = 0.0059), however there
288 was no apparent olfactory discrimination between S. campanulata or K. africana for
289 male beetles (Fig. 3E, n = 9, P = 0.5000), indicating a gender-based response to
290 olfactory cues from the host plants.
291 The latency period did not differ for S. campanulata or K. africana ($F_{1,28} = 0.39$, P =
292 0.5390). However, latency was significantly greater for male than for female P.
293 coerulea ($F_{1,28} = 5.7320$, P = 0.0239).
294
295 3.4 Conspecific Cues
296 There was a significant difference in the number of times P. coerulea was observed
297 in the vial containing treated filter paper with respect to the gender combination
298 treatment ($H_{3,120} = 25.18$, P = <0.0001). Significant deviation from the expected N_T =

4, was observed for the female response to male and male response to other male treatments (Table 2). Females were appreciably attracted to male cues (P = <0.0001), whereas male-treated filter paper deterred other male beetles (P = 0.0107). There was no apparent response to female cues by either other females (P = 0.4203), or by males (P = 0.1694).

4. DISCUSSION

Adult P. coerulea will feed on the leaves of both S. campanulata and K. africana, but larval development is not possible on K. africana. Acceptability for oviposition was 12 times greater on S. campanulata than for K. africana in paired choice tests, and in sequential no-choice tests larvae were only ever found on S. campanulata. Adult feeding on S. campanulata was approximately four times greater than on K. africana, indicating a clear preference for the target plant. Non-target feeding on K. africana was recorded under both choice and no-choice conditions, suggesting that a spill-over effect might occur in the field if populations of P. coerulea built-up on S. campanulata growing in close proximity to K. africana, although the relative performance was so poor on K. africana that significant levels of attack are unlikely (see Paynter et al., 2015).

Paradibolia coerulea utilises olfactory cues to help select its typical host plant. This has also been shown to be the case for many other Chrysomelidae (McIndoo, 1926; Metcalf & Metcalf, 1992; Muller & Hiker, 2000; Hori, Ohuchi & Matasuda, 2006; Fernandez & Hilker, 2007). There was a preference for cues emitted by S. campanulata over both control and K. africana containing-stems, but this was limited to female individuals. Male beetles demonstrated no olfactory discrimination between host plants and had a significantly greater latency period before selecting either host.

During choice experiments there were several instances where eggs or larvae were evident on K. africana, yet in sequential no-choice assays there were no eggs on K. africana plants, which could indicate that oviposition-stimulating volatiles from S. campanulata accumulated in the paired-choice cages and this resulted in some oviposition on the atypical host. Our findings indicate that if female P. coerulea are unable to locate S. campanulata, there is little risk of oviposition on K. africana.

Another factor that may contribute to host plant selection by insects is that of conspecific herbivore chemical emissions (Tansey et al., 2005; Fernandez & Hilker, 2007). There is substantial evidence among chrysomelid beetles for roles in attracting conspecifics by both female-emitted sex pheromones (Cuthbert & Reid, 1964; Zhang & McEvoy, 1994) and male-emitted aggregation pheromones (Peng, Bartelt & Weiss, 1999; Dickens et al., 2002). Our results demonstrate a strong attraction to male-emitted cues by conspecific female P. coerulea individuals, while males were deterred by the presence of other male cues. Whether the individuals used in the study had mated was unknown, and it is possible that the response to conspecifics could change after mating. Tansey et al. (2005) obtained a similar result with female Aphthona nigriscutis Faudras (Chrysomelidae) orientating to male-emitted cues. Wood (1982) proposed that this male-attract-female synergism for Dendroctonus frontalis Zimmermann (Coleoptera: Chrysomelidae) allowed for host plant selection by the male, which would then attract female conspecifics. Our results indicate that male P. coerulea are unable to discriminate between the two plants but that the females are attracted to the preferred host, S. campanulata. Females are required to locate their host as they select plants for oviposition and the larvae can survive on S. campanulata, but not K. africana. Both sexes can feed on either species as adults so there may be no selection pressure for males to be attracted to S. campanulata specific volatiles.

Females are also apparently required to seek out males for mating as females showed an attraction to male beetles but no attraction of males to females was recorded. This suggests that another cue, such as a visual or tactile cue, attracts males to S. campanulata plants under natural conditions. Alternatively, males may only be attracted to virgin females and most of the 12-15 day old beetles used in the trials may have been mated.

The different response of the sexes to conspecifics, as well as to the host plant, should be considered when further host specificity testing is conducted. If choice tests are required, it is important that the sex of the beetles that are used in the tests are known. If male beetles are used, then they are likely to feed indiscriminately on all close relatives of S. campanulata because they are not attracted by the volatiles of the primary host. If females are used in the same tests, a much higher proportion of feeding on S. campanulata would be expected. The presence of males may also influence which plants female beetles feed on, because females are attracted to males. A higher proportion of males in a choice experiment could therefore result in greater levels of feeding on non-target plants.

The results of the sequential no-choice tests suggest that female adult feeding on S. campanulata is required for the production of eggs. P. coerulea was capable of egg production if newly emerged beetles had fed on S. campanulata (in the ABA treatment) but not when the newly emerged beetles had fed on K. africana (in the BAB treatment). Egg production was also greatest in the AAA treatment where the beetles had the longest period of exposure to S. campanulata. A similar effect was observed in host specificity testing of the leaf-mining fly, Phytomyza vitalbae Kaltenbach (Agromyzidae), a biological control agent for Clematis vitalba L. (Ranunculaceae), which produced fertile eggs on closely related non-target species, but only when adults

had previously fed on the target plant (Schwarzländer, Hinz & Wittenberg, 1996). Adult P. vitalbae that had not fed on C. vitalba were infertile and spill-over onto non-target species in the field was therefore limited (Paynter et al., 2008). Non-target damage in the field was usually restricted to an area within 4km of populations of the target plant and was never found further than 30km from a C. vitalba population (Paynter et al., 2008). A similar spill-over effect on K. africana would be expected if P. coerulea were released in a region were both S. campanulata and K. africana occur.

This study demonstrates how investigations into the effect of olfactory cues can be useful in elucidating the host range and behaviours of potential biological control agents. In this case, P. coerulea prefers S. campanulata and cannot complete its development on K. africana, but some spill-over onto K. africana may occur if the insect were released in areas where the two plants grow together. Any damage to K. africana would be a temporary spill-over at times when abundance of P. coerulea on S. campanulata was high. Further host specificity testing, using other test plants in the family Bignoniaceae and from closely related families, is required before a full assessment of the host range of P. coerulea or decisions about the safety of the beetle as a biological control agent can be made, but the design and interpretation of these host specificity tests should take the olfactory responses of P. coerulea to both the host plant and conspecifics into consideration.

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Legend to figures

Fig. 1. Schematic overview of sequential no-choice test design for host-range determination of Paradibolia coerulea.

Fig. 2. Feeding (a) and reproductive (b) responses of P. coerulea pairs to sequential presentation of Spathodea campanulata (A) and Kigelia africana (B) in an ABA and BAB sequence, with a control employed in an AAA sequence. Bars indicate a mean (± SD), with grey bars for S. campanulata and white bars for K. africana. An asterisk indicates a value of zero. Five replicates were conducted per plant sequence.

Fig. 3. Preference responses (%) of Paradibolia coerulea to olfactory cues emitted by Spathodea campanulata (SP) and Kigelia africana (KI) leaves, in a stem-arena. Controls (CT) had no plant material placed inside a stem. Treatments were: (A) SP/CT, (B) KI/CT, (C) SP/KI, (D) SP/KI (female beetles) and (E) SP/KI (male beetles). Sample sizes (n) and P-values (P) are provided for each treatment, with significant preferences following binomial tests indicated by an asterisk.