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Honeybush (*Cyclopia* spp.) mature embryo culture and the influence of pod position on seed colour

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Plants (different genotypes) of *Cyclopia,* used for production of South African honeybush tea (a herbal tisane product), are being improved through breeding for large-scale cultivation, but seed dimorphism and dormancy negatively affect germination percentage. In order to increase the percentage of plants that can be obtained from a batch of seed, mature embryo culture and the influence of pod position on seed colour were investigated for *C. longifolia*, *C. maculata* and *C. subternata*. Mature embryos were excised and cultured *in vitro* at 25 °C ± 1 °C, 24-hour photoperiod, with and without prior cold treatment. The culture of mature embryos proved to be very successful, consistently resulting in more plantlets than conventional sowing. Mature honeybush pods were also collected from the upper, middle and lower-third foliar portions of honeybush shrubs to determine the number of green/indeterminate/brown seeds produced in each position. However, the ratio of dimorphic seed colours produced could not be predicted by pod position.

Keywords: embryo culture, *in vitro*, dormancy, cold treatment, seed dimorphism

Heuningbos (*Cyclopia* spp.) volwasse embrioweefselkultuur en die effek van peulposisie op saadkleur: Plante (verskillende genotipes) van *Cyclopia* spesies word gebruik om Suid-Afrikaanse heuningbostee ('n kruietee produk) te produseer en word tans deur teling vir grootskaalse verbouing verbeter. Saaddimorfisme en dormansie beïnvloed egter die ontkiemingspersentasie negatief. Deur gebruik te maak van volwasse embrioweefselkulture is studies om die ontkiemingspersentasie van saad te verhoog op *C. longifolia*, *C. maculata* en *C. subternata* gedoen. Die effek van stratifikasie op saadontkieming deur geen of 'n kouebehandeling toe te pas, is ook ondersoek. Volwasse embrios is hierna uit sade verwyder en vir 'n 24-uur fotoperiode by 25°C ± 1°C op weefselkultuur gekweek. Die kweek van volwasse embrios deur middel van weefselkulture was suksesvol en het deurlopend meer plante vergeleke met konvensionele saaimetodes gelewer. Studies om die effek wat saadpeulposisie van die moederplant op saadkleur het, is ook gedoen. Volwasse heuningbospeule is vanaf die boonste, middelste en onderste-derdes van die blaarbedekte deel van die heuningbosplante versamel om die aantal groen/intermediêre/bruin sade wat by elke gedeelte geproduseer is, te bepaal. Die verhouding van saaddimorfisme wat by elke gedeelte geproduseer word, kon egter nie deur peulposisie voorspel word nie.

Sleutelwoorde: embrioweefselkultuur, *in vitro*, kouebehandeling, saaddimorfisme, saaddormansie

Introduction

The genus *Cyclopia* (Family Fabaceae) is indigenous to the fynbos areas of South Africa. There are 23 recognised species within the genus, all woody shrubs of various sizes, of which a handful are grown commercially for the production of "honeybush tea" (Schutte, 1997). The small, but growing, honeybush industry exports about 390 tons annually, with an export value of R23 million (Department of Agriculture Forestry and Fisheries, 2016; McGregor, 2017). Issues with the cultivation and breeding of honeybush have sparked a number of research studies in support of the industry. The aim of the present study was to increase the number of plants that can be obtained from a batch of honeybush seed. Questions about seed dormancy, the viability of mature seed culture as an alternative to conventional sowing for small samples, and seed colour as associated with pod position on the plant, are investigated.

In legumes, the ovary elongates and develops into a dry fruit called a pod after fertilisation of the ovules. Within the pod, fertilised honeybush ovules develop into small (3–5 mm in length)

seeds with three genetically distinct components: the embryo, the endosperm and the seed coat (Koen et al., 2017). Honeybush species produce mature seeds with brown and/or green colour seed coats, with both colours often produced in the same pod (M. Motsa, pers. comm., 2015). A previous study found that differences in *Cyclopia* spp. seed colour correspond to differences in germination percentage under various treatments (Koen et al., 2017). Brown seeds were found to be physically less dormant and more susceptible to fatal damage from scarification treatments than green colour seeds. The factors affecting the production of different seed coat colours have hitherto not been identified for honeybush but maternal environmental factors have been shown to influence the dimorphic seed production of other species (Cheplick and Sung, 1998; Wang et al., 2008). If seed colour can be predicted by pod position, then combining pods collected from the same position would result in increased seed lot uniformity.

Honeybush seeds have either physical or combinational dormancy, depending on the species in question (Baskin and Baskin, 2004; Koen et al., 2017). This dormancy must be overcome before seed germination can take place. Excision and culturing of mature honeybush embryos *in vitro* before seed ripening and desiccation take place would effectively circumvent physical dormancy and possibly physiological dormancy as well (Debeaujon et al., 2018). The presence of these dormancies, and the losses incurred through dormancy-breaking treatments, contribute to poor seed germination percentages in honeybush (Koen et al., 2017).

Physiological dormancy may be endogenous (caused by factors within the embryo) or exogenous (imposed upon the embryo by factors such as inhibitory substances within the seed coat) relative to the embryo (Baskin and Baskin, 2004). If physiological dormancy in honeybush is exogenous, then removing the embryos from their surrounding tissues (seed coat layers, including endosperm) should bypass it and eliminate the need for dormancy-breaking treatments (such as cold stratification). On the other hand, if embryos excised from hydrous seeds (Fig. 1.a) do not display physiological dormancy, and embryos excised from desiccated seeds (Fig. 1.b) do, it may be an indication that endogenous physiological dormancy develops within the embryo during or after the desiccation stage of seed maturation.

Physiological dormancy has commonly been associated with the "resprouter" fire-survival strategy among honeybush species, as contrasted to the "seeder" fire-survival strategy (Whitehead & Sutcliffe, 1995; Joubert et al., 2011; Wooldridge et al., 2012; Koen, 2015; Brink et al., 2017). Schutte (1997) described fire-survival strategy, or the preference of producing many seeds (so-called seeder species) versus producing a woody, subterranean rootstock (sprouter species), as a useful distinguishing characteristic between honeybush species. However, this characteristic is known to be polymorphic within honeybush species (Schutte, 1997; Joubert & Joubert, 2012). *Cyclopia longifolia*, *C. subternata* and *C. maculata* have been labelled as seeder species (Schutte, 1997; Joubert et al., 2011), but Joubert and Joubert (2012) indicated that *C. longifolia* may act as a seeder and/or sprouter. Schutte (1997) deliberately did not include sprouting/non-sprouting in cladistic analyses, because it is a "polymorphic character" – present in some populations while absent in others.

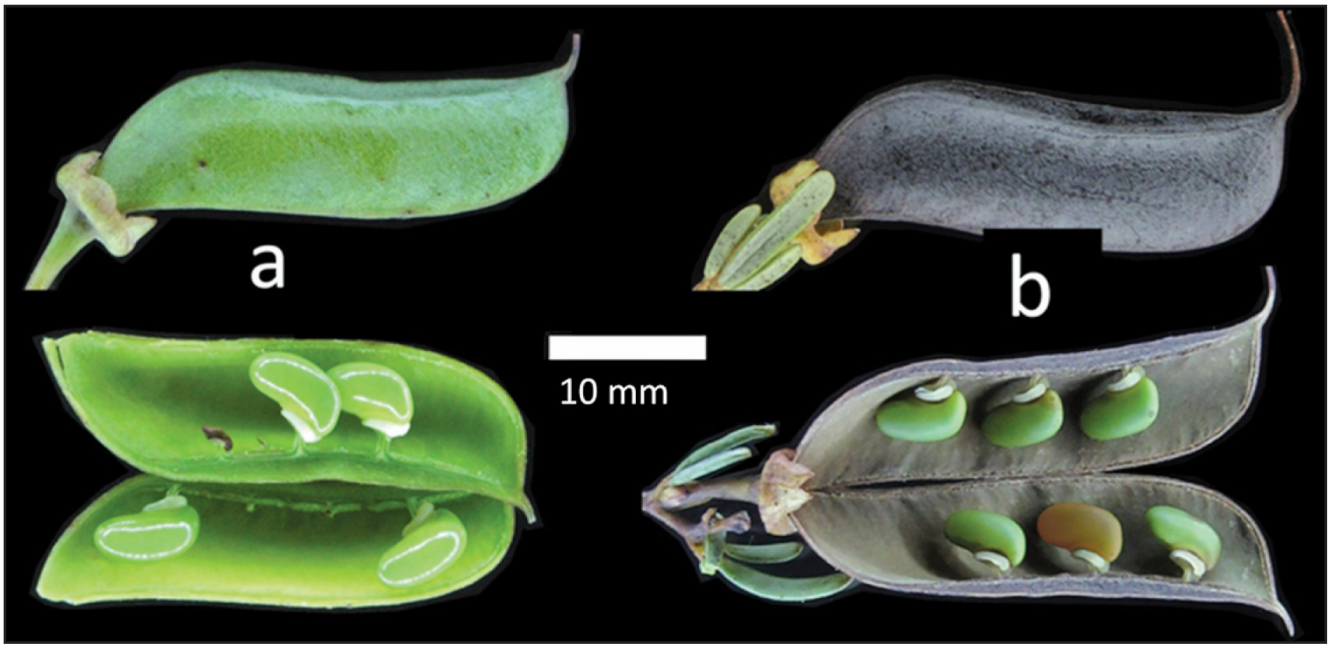

FIGURE 1: *Cyclopia subternata* pods; a = prior to natural desiccation (hydrous seeds); b = following natural desiccation (desiccated seeds)

All previously tested honeybush species have been shown to benefit (in terms of germination rate and percentage) from a period of cold treatment following imbibition and prior to incubation or sowing (Koen et al., 2017; Whitehead and Sutcliffe, 1995). Physiological dormancy has, however, not been consistently reported for all honeybush species.

Material and methods

All the plant materials used in the experiments were collected from open-pollinated, honeybush seed orchards at Nietvoorbij Research Farm (S 33° 54′ 23.417′′ E 18° 52′ 14.196′′), near Stellenbosch in the Western Cape province of South Africa. Samples were taken from three species, *C. longifolia*, *C. maculata* and *C. subternata*, between July and October of 2017. All chemicals and laboratory consumables were sourced from Sigma-Aldrich, Inc.

Mature embryo culture

Collection of plant materials

Each seed sample was collected from several individual plants of the same clone, except for the experiment with desiccated seeds from *C. maculata,* in which case a mix of various clones was used. Both hydrous and desiccated seeds were collected from *C. longifolia* from clone LHK8. From *C. maculata*, hydrous seeds were collected from clone MBV4 and desiccated seeds from an unspecified mix of *C. maculata* clones. From *C. subternata*, both hydrous and desiccated seeds were collected from clone SKB6. In order to obtain embryos from two seed types, 1) mature-hydrous, and 2) mature-desiccated, pods were harvested just before, and immediately after, the natural process of pod maturation had taken place (Fig. 1.a-b). Desiccated seeds within a sample were assumed to have all reached the same level of maturity. Seeds were obtained from open-pollinated source plants and the number of days having elapsed after pollination could not be established, therefore maturity of the hydrous seed could not be precisely equal. In order to ensure a uniform test result between hydrous seeds, a sample seed was chosen for comparison from each species. If the contained embryo was found to be fully mature (at the last stage of development before desiccation), the sample seed was used as a visual guide and hydrous seeds with similar morphological characteristics were selected for that species for the remainder of the experiment. Ten embryos were excised for each replication, with four replications per treatment.

Seed treatment

Desiccated seeds required scarification and rehydration prior to embryo excision and this was done prior to surface sterilisation. Scarification was done by soaking the seeds in concentrated (95%) sulphuric acid, followed by three water rinses. Acid exposure time was one hour for *C. longifolia*, 45 min for *C. maculata* and one hour for *C. subternata* (Bester and Koen, 2017). The desiccated seeds were then allowed to rehydrate by being soaked in water for six hours. Hydrous seeds were surface sterilised directly after being removed from the pods, with no additional treatments. Surface sterilisation for both seed types was the same, viz the seeds were placed under running water for 15 minutes, followed by a five minute soak in 1% sodium hypochlorite solution. The seeds were then rinsed three times in sterilised water before being rinsed briefly (about 1 min) in 70% ethanol, followed by three final rinses in sterilised water. Embryos from each seed type were then excised under sterile conditions in a laminar flow cabinet.

Culture media

Embryos were placed on Murashige and Skoog (1962) basal medium; Sigma-Aldrich, Inc. product code M5524. The basal medium was prepared at half concentration for use throughout the study, meaning that every 4.3 g of powder was combined with two litres of water instead of one litre water. This was done in order to promote root growth (Dewir et al., 2016; Kokotkiewicz et al., 2012; Pratap et al., 2010; Sánchez-Romero et al., 2007; Smith, 2013). The premixed medium included both salts and vitamins, it was prepared with plant tissue culture agar (0.8% w/v), sucrose (1.5% w/v) and activated charcoal powder (0.8% w/v). Media pH was adjusted to 5.6 prior to autoclaving. Media were set in polystyrene Petri dishes (95 mm), eight dishes per medium, about 30 ml per dish. Once the embryos had been excised and placed on the medium, the Petri dishes were sealed with Parafilm®.

Incubation conditions and data collection

Half of the dishes were set directly into a temperature-controlled incubator at 25 °C ± 1 °C, 24-hour photoperiod, 35 µmol m^{-2} s^{-1} light intensity. The remaining dishes were placed into a refrigeration unit at 4 °C in darkness for two weeks prior to being moved to the incubator (cold stratification treatment). Each Petri dish contained 10 embryos and represented one replicate. For embryos placed directly in the incubator, day 7 represented the first observation day. For embryos that were cold treated prior to being moved to the incubator, the first day of being placed in the incubator (day 0) was the first observation day because it was anticipated that some embryos may already have begun to germinate during the two-week cold treatment (Koen et al., 2017). Once root+hypocotyl length ≥ 10 mm and the cotyledons had opened, embryos were counted as germinated and removed from the Petri dishes into 180 ml culture bottles prepared with the same, half-strength MS basal media formulation (about 30 ml per bottle) for continued growth.

Positive control

Two hundred seeds each from *C. longifolia* (LHK8), *C. maculata* (various clones from 2017) and *C. subternata* (SKB6) were sown in separate trays to compare the success rate of embryo culture with conventional sowing. Growth media

formulation and acid scarification were according to the Agricultural Research Council's honeybush propagation guidelines for seedlings (Bester and Koen, 2017).

Statistical procedure

The experimental layout was a completely randomised design. Six trials (three species by two seed types) were combined after testing for variance homogeneity (Levene, 1960). Each trial comprised of a split-plot where medium was the main plot and the stratification treatment the subplot. The data were recorded as percentage germination and subjected to a combined analysis of variance using the General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

Influence of pod position

Collection of plant materials

In order to investigate the effect of pod position on seed colour dimorphism, mature but undehisced honeybush pods were collected from open-pollinated seed orchards. Fifteen pods were collected from, respectively, the lower, middle, and upper-third sections of three clones of two honeybush species. The *C. longifolia* clones used were LGR2, LHK23 and LHK35. The *C. maculata* clones used were MBV3, MBV4 and MBV10. Five pods were used per replicate with three replicates per clone. Unfortunately, the species *C. subternata* could not be included in this study because the ripened pods were accidentally collected by labourers and indiscriminately mixed. After the pods were shelled, the seeds were pooled within pod position and replicate while being kept separate between clones or species. The seeds were then divided into three colour groups (green, brown and indeterminate) and counted.

Statistical procedure

The experimental design was a completely randomised design with three replications, two species and three clones per species. The treatment design within a species was a split-split plot. The main plot factor was the clones. The first subplot factor was the position of the seeds (lower, middle and upper-third sections of the bushes) and the second subplot was the colour of the seeds (green, brown and indeterminate). The Levene test showed heterogeneous (not comparable) species variances (Levene, 1960). Therefore the data was subjected to a weighted combined analysis of variance using the General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). The weights were the reciprocal of the variance of each species (John and Quenouille, 1977). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

Results

Mature embryo culture

Results of the conventionally sowed seed (positive control) were 75% germination for *C. longifolia*, 38% for *C. maculata* and 70% for *C. subternata* (no figure given).

Species

Small, though significant, differences were observed in the number of embryos that developed and those that succumbed to microbial contamination (died) (Developed: Least Significant Difference $(LSD)_{p=0.05}$=4.9; Died: $LSD_{p=0.05}$=5.6) (Fig. 2). No significant differences were observed in the number of embryos that remained undeveloped between the three species. *Cyclopia maculata* and *C. longifolia* had the highest percentages of embryos that germinated with no significant difference between the two species. Although there was no significant difference between *C. subternata* and *C. longifolia*, significantly fewer *C. subternata* embryos germinated compared to *C. maculata*. Overall, the number of plantlets obtained by embryo culture was greater than the positive control by 24% for *C. longifolia*, 56% for *C. maculata* and 18% for *C. subternata*.

Cold treatment

There were no significant differences between species in response to cold treatment. Pooling the species for a total of 120 embryos per treatment, significant differences were observed in the number of embryos that developed, those that died, and those that remained undeveloped, in the interaction between treatment and embryo type (Developed: $LSD_{p=0.05}$=3.6; Died: $LSD_{p=0.05}$=3.7; Remained undeveloped: $LSD_{p=0.05}$=1.5) (Fig. 3). In both treatments, significantly greater percentages of developed embryos were obtained with hydrous embryos compared to desiccated embryos. There was no significant difference between the results for hydrous embryos in both treatments (Fig. 3). For desiccated embryos, the cold treatment resulted in significantly fewer plantlets compared to the treatment that omitted cold stratification.

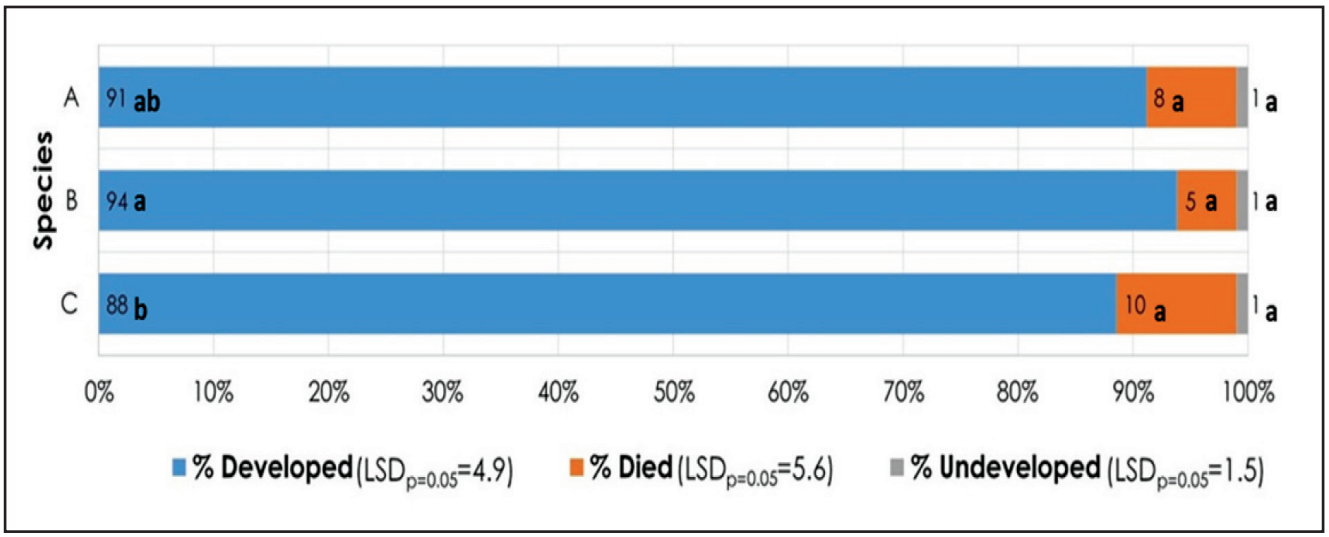

FIGURE 2: Percentages of *Cyclopia* spp. embryos that developed, died or remained undeveloped, according to species. A = *C. longifolia*; B = *C. maculata*; C = *C. subternata*. Means within the same colour with the same letter are not significantly different at p = 0.05. Values rounded to the nearest integer.

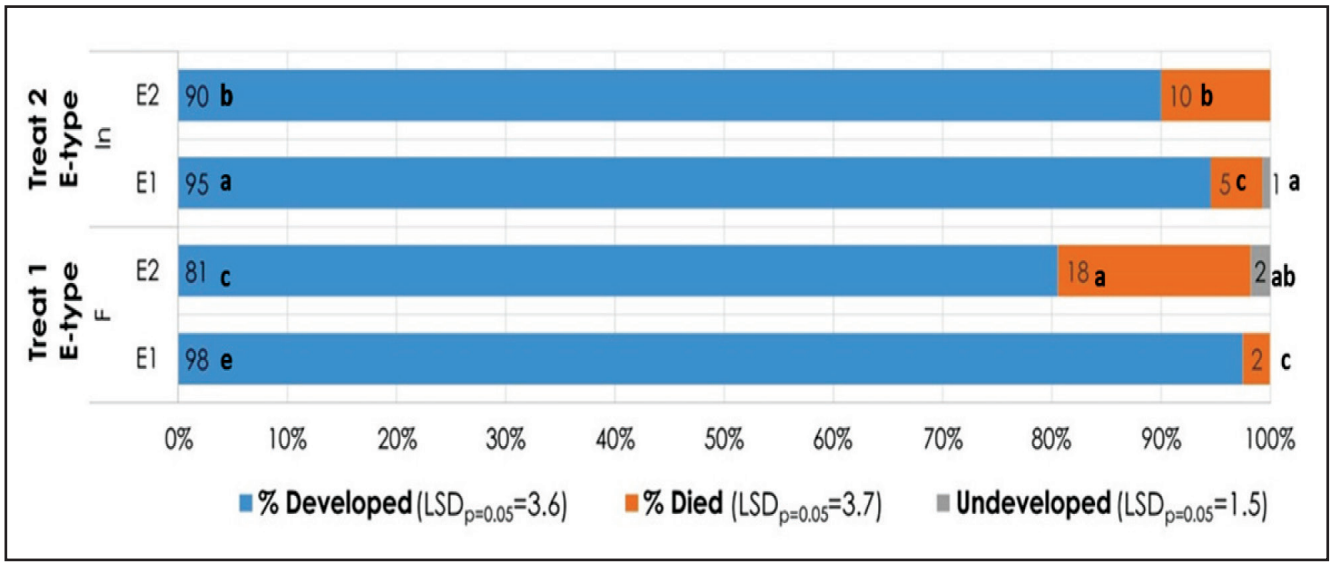

FIGURE 3: Percentages of *Cyclopia* spp. embryos that developed, died or remained undeveloped, according to treatment and embryo type. Treat 1 = cold treatment; Treat 2 = without cold treatment; E-type = embryo type; E1 = Hydrous embryos; E2 = Desiccated embryos. Means within the same colour with the same letter are not significantly different at p = 0.05. Values rounded to the nearest integer. Null values omitted.

As expected, a number of embryos (approximately 5%) were observed to begin germination during the cold stratification treatment. It was additionally observed that even embryos which did not begin germinating during the cold treatment increased noticeably in size while retaining their embryonic proportions. Upon being moved to the incubator, these embryos developed rapidly.

Influence of pod position

Mean numbers of seeds collected over species, pod position and seed colour

The mean number of seeds collected per species, over all other factors, was 11.84 for *C. maculata* and 6.77 for *C. longifolia*. The species *C. maculata* was observed to produce significantly more seeds per pod than *C. longifolia* (a difference of 27%) ($LSD_{p=0.05}$=1.03) (Fig. 4). Pod position did not seem to have a great influence on the number of seeds produced. The mean number of seeds collected for pod position, over all other factors, was 8.54 for the upper position, 8.15 for the middle position and 7.34 for the lower position. Pods taken from the upper position did have more seeds than those taken from the lower position, but the difference, though statistically significant, was small (3%) ($LSD_{p=0.05}$=0.96) (Fig. 4). The mean number of seeds collected per seed colour, over all other factors, was 16.82 for green, 5.69 for brown and 1.52 for indeterminate. Overall, significantly more green colour seeds (70%) were produced than brown (24%) or indeterminate seeds (6%) ($LSD_{p=0.05}$=1.33) (Fig. 4).

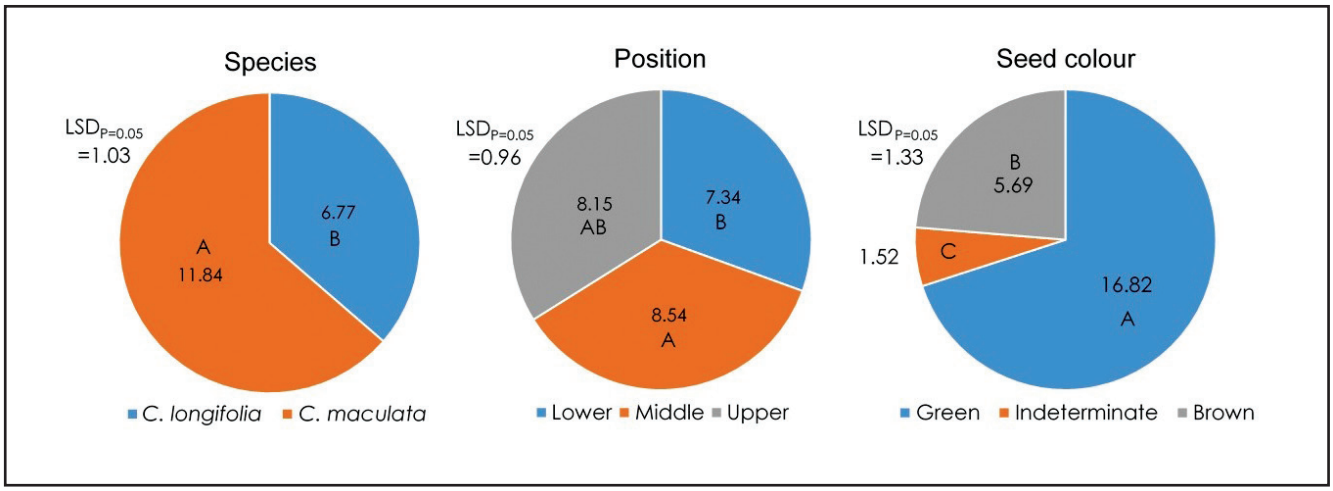

FIGURE 4: Mean number of *Cyclopia* spp. seeds collected from five pods, calculated for species, position and seed colour, respectively. Means within the same figure with the same letter are not significantly different at p = 0.05.

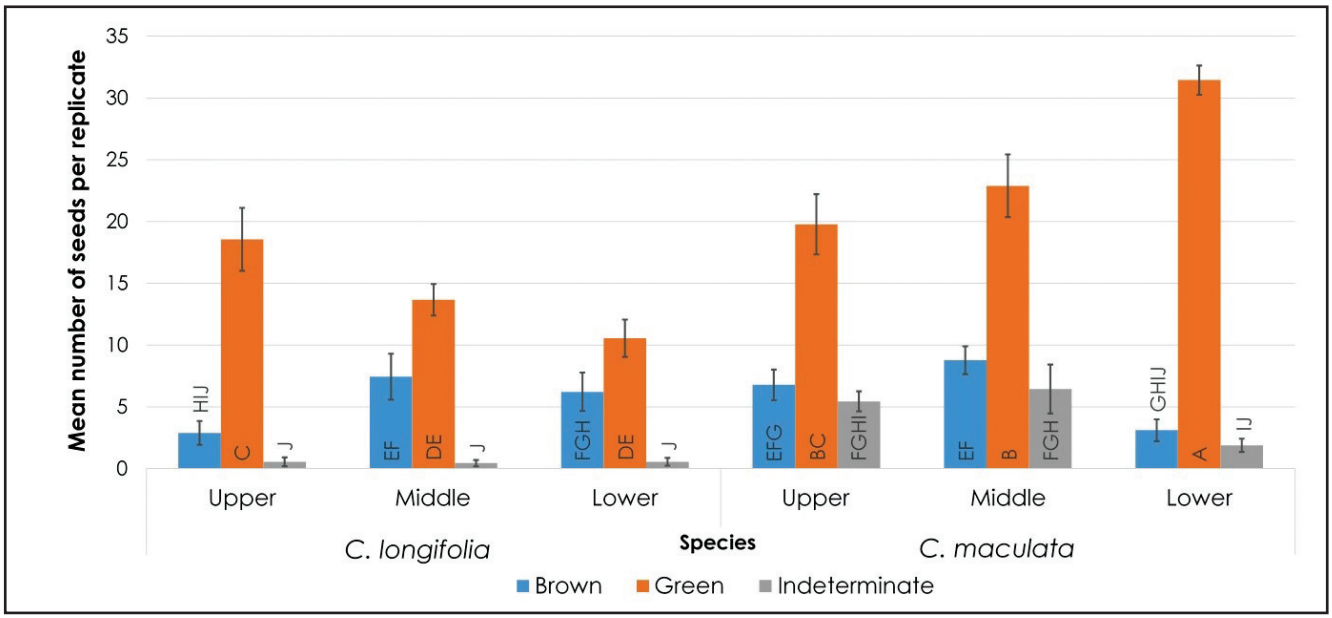

FIGURE 5: Mean number of *Cyclopia* spp. seeds collected per replicate (five pods), combined interaction between species, pod position and seed colour. Means with the same letter are not significantly different at p = 0.05.

Species compared

Statistically significant interactions were detected between pod position, seed colour, species and clones within species. The average over clones for the species showed that more green colour seeds tend to be produced than brown or intermediate colour seeds at all pod positions (Fig. 5). The only other apparent similarity in the pattern of interaction for pod position and seed colour between the species was a slight tendency for a greater number of brown seeds to be produced in the middle position for both species (Fig. 5).

Clones compared

A great deal of variation in the ratio of dimorphic seeds produced at different positions was observed between clones for both species (Fig. 6-7). No consistent pattern of seed colour distribution in relation to pod position was found between clones within the species. The greatest number of green colour seed was collected from the upper position in two of three clones for *C. longifolia* (Fig. 6), and from the lower position in two of three clones for *C. maculata* (Fig. 7).

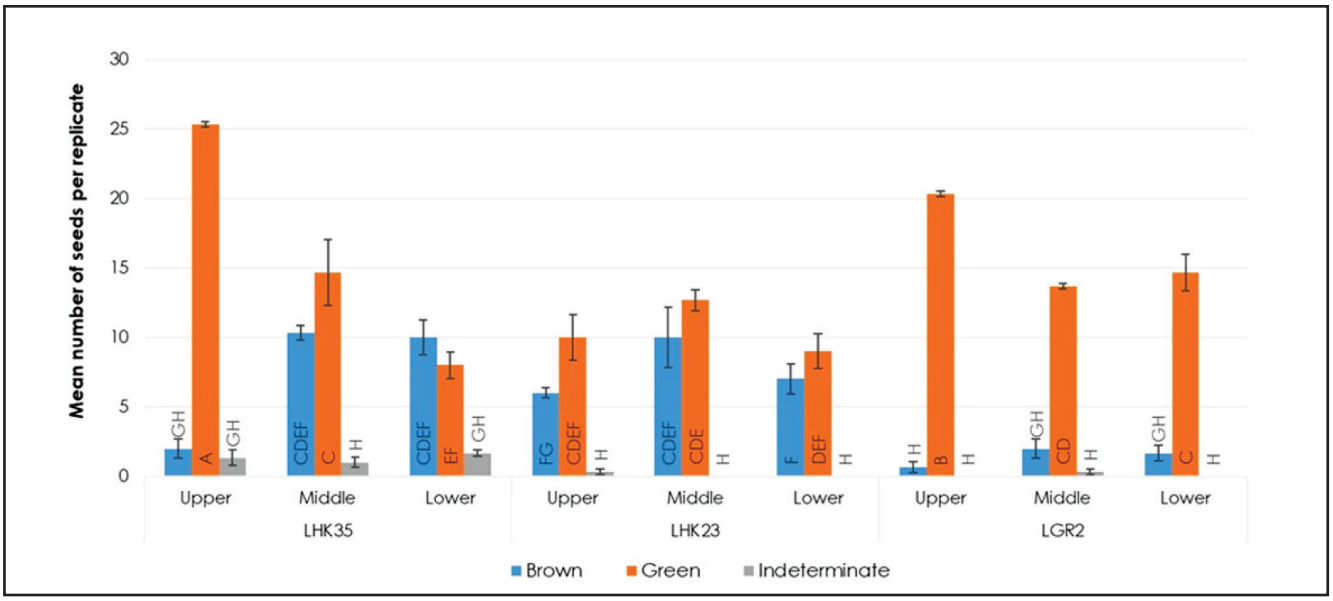

FIGURE 6: Mean number of *Cyclopia longifolia* seeds collected per replicate (five pods), combined interaction between species, pod position and seed colour. Means with the same letters are not significantly different at p = 0.05.

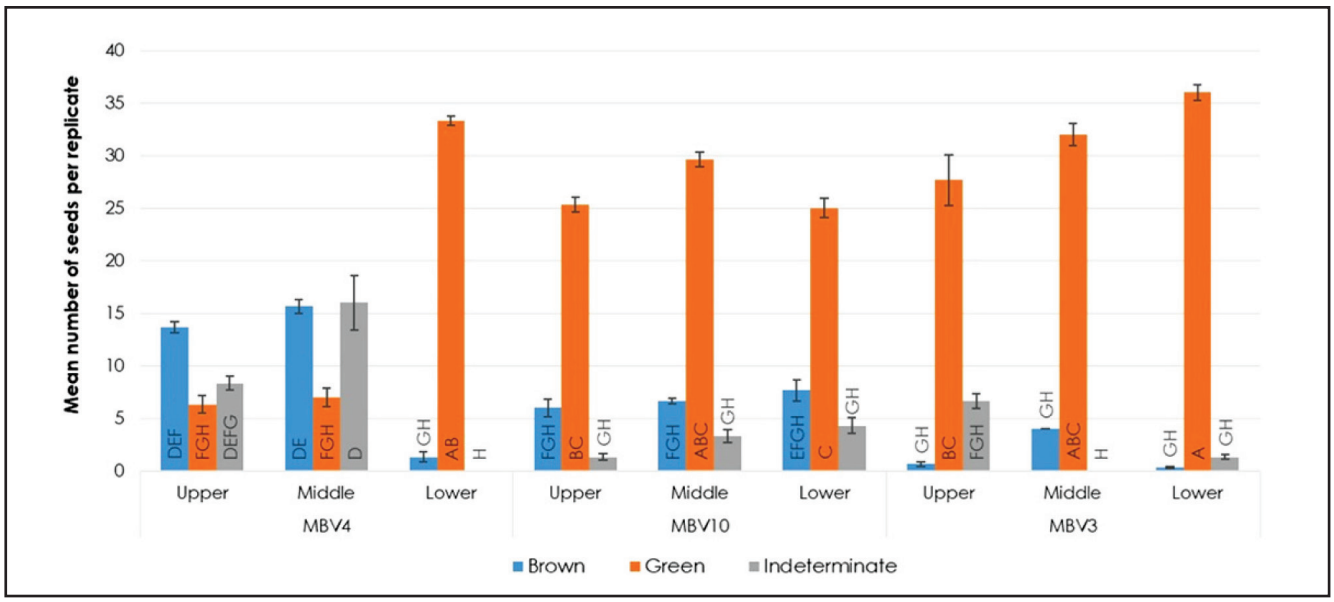

FIGURE 7: Mean number of *Cyclopia maculata* seeds collected per replicate (five pods), combined interaction between species, pod position and seed colour. Means with the same letters are not significantly different at p = 0.05.

Discussion

Mature embryo culture

Species

Though considered to be a seeder species (Schutte, 1997), the low percentage of seedlings resulting from the conventional sowing of *C. maculata* seeds (38%) may be an indication of some degree of seed physiological dormancy being present in the tested batch of seed. When comparing the percentage of seedlings to the percentage of plantlets that developed from excised *C. maculata* embryos (94% see Fig. 2), the difference between the two methods is stark. This suggests that physiological dormancy in honeybush seeds may be exogenous relative to the embryo – i.e. imposed by the seed coat and/or endosperm tissues. It should be remembered that a single clone (MBV4) was used in the culture of embryos from hydrous seeds while various clones from harvest year 2017 were used in the culture of embryos from desiccated seed and in the conventional sowing trial. It is possible that genotypic variation between *C. maculata* clones may have had some effect on the final result. However, the percentage of plantlets that developed in culture from the mixed-clone *C. maculata* seed sample was still much greater than the percentage of seedlings obtained from conventional sowing i.e. the positive control. In the conventional sowing trial, germination percentages for *C. longifolia* and *C. subternata*

were relatively high, as may be expected of seeder species (Motsa et al., 2017; Schutte, 1997). Nevertheless, the number of plantlets obtained through mature embryo culture was also higher than the number of seedlings obtained through conventional sowing for these two species.

Cold treatment

No significant differences were observed between the treatment types overall, nor any in the interaction with species. It seems that cold stratification of excised honeybush embryos is unnecessary, although perhaps useful as a conditioning treatment, because physiological dormancy was circumvented by the removal of embryos from their seed coats/endosperms. Future studies may confirm these findings with other honeybush species, such as *C. intermedia*, which are known to produce physiologically dormant seeds (Whitehead and Sutcliffe, 1995).

Influence of pod position

The purpose of the present study was to determine whether seeds could be separated according to colour during harvest simply by grouping pods collected from different elevations on the plant. Unfortunately, although statistically significant tendencies were observed (Fig. 6-7), the effect is not great enough to make a practical difference to the problem of seed colour separation. Genetic variables are likely to be a significant factor in determining behavioural differences in this genus of polyploid species (*Cyclopia*), but maternal field environment has also been linked to divergent seed characteristics in other species and must be duly considered (Carta et al., 2016; Gutterman, 2000; Motsa et al., 2018; Penfield and MacGregor, 2016; Postma and Ågren, 2015; Wulff, 2017). Environmental factors (i.e. local climate) which can have an effect on seed development may include photoperiod and light intensity, altitude, water stress, and especially temperature (Carta et al., 2016; Gutterman, 2000; Penfield and MacGregor, 2016). According to Penfield and MacGregor (2016), even slight differences in maternal and/or environmental factors may result in seed dimorphism, because the seed coat is a "highly plastic plant organ", i.e. very responsive to environmental signals (whether mediated by the maternal genotype or acting directly upon the zygote). Recent studies on select honeybush species have begun to reveal details about flower structure and pollen viability, which may also have an effect on pollinator interactions and, consequently, successful fertilisation (Koen et al., 2020a; Koen et al., 2020b). It is probable that a complex combination of the abovementioned factors is responsible for determining the highly variable ratio of dimorphic seed produced in open-pollinated, field grown honeybush stands. Future studies may investigate the effect of these interactions on ratio and distribution of brown and green colour seeds in honeybush. Currently, there are still too many unknowns to make an educated guess on what the primary causal factors may be.

Conclusion

In the embryo culture experiment, *in vitro* culture consistently resulted in higher percentages of plantlets than conventional sowing and may be a viable alternative to germination for small samples. Seed dormancy was successfully circumvented by removal of the seed coat, rendering cold treatment unnecessary. This suggests that physiological dormancy may be exogenous to the embryo, but further study for the sake of disambiguation is recommended. The use of hydrous seeds for mature embryo culture was more successful than desiccated seeds and is recommended. Hardening-off protocols urgently need to be developed for plantlets resulting from mature embryo culture. In the dimorphic seed study, it was found that the ratio of dimorphic seed colours produced is not greatly affected by pod position. Therefore, unfortunately, obtaining seed lots of a more uniform colour will not be as simple as merely adjusting the harvesting method. The factors determining seed coat colour in honeybush species remain to be determined.

Acknowledgements

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