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Full Length Research Paper

Factors affecting *in vitro* degree of browning and culture establishment of pomegranate

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The present study was conducted to identify the most suitable types of nodal explants and browning control treatment for *in vitro* regeneration of pomegranate. Murashige and Skoog (MS) medium containing 1.0 mg/L BAP + 0.5 mg/L NAA was used commonly for all the treatments tested. Result revealed that the intensity of browning was increased with increased position and the length of explants. Minimum browning intensity was observed in 1st nodal explants having 1.5 cm length. However, explants of 3rd node with 2.5 cm length registered higher establishment (68.5%) and growth of explants. Furthermore, the most effective browning control was observed in subculturing of nodal explants twice, at the first day and third day of inoculation, which also found better in establishment of explants followed by activated charcoal 200 mg/L into the medium. Maximum length of shoots (3.9 cm) was recorded in 1st position of node with 2.5 cm length of explants.

Key words: Nodal segments, position, antioxidants, browning, establishment.

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae. Pomegranate is widely grown in many tropical and subtropical countries, especially in moderate climate of meditation region (Salaheddin and Kader, 1984). Generally, cultivation of pomegranate is done by using vegetative propagated (hardwood cutting and air layering) plantlet for the field planting. However, the conventional propagation methods of pomegranate are not found suitable to provide large-scale of planting material at a time, as it is rather slow for multiplication of plants. Consequently, the availability of planting materials is restricted throughout the year. Tissue cultured plants are more advantageous than those by conventional propagation (Moore et al., 1991). Moreover, *in vitro* techniques are one of the reliable sources used for commercial plantlet production of pomegranate. *In vitro* propagation of woody plants is recalcitrant for growth because of browning problem at initial establishing stage of *in vitro* culture (Zaid, 1984; Pirttila et al., 2008; Krishna et al., 2008), due to leaching of phenolic substances and secondary metabolites from cut surface which hamper further morphogenesis response and rooting of explants (Aliyu, 2005). Explants and medium browning is a major problem in pomegranate due to the exudation of high

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amount of phenols, especially in mature explants (Naik and Chand, 2010).

Phenols are chemical compounds that embraces a wide range of plant substances which posses in common, an aromatic ring bearing one or more hydroxyl constituents (Onuoha et al., 2011). Various attempts has been made to multiply pomegranate by using tissue culture techniques through shoot tip and nodal segment explants of mature plant (Kantharajah et al., 1998; Singh and Khawale, 2006; Kanwar et al., 2009; Samir et al., 2010). However, the problem of browning and death of culture during *in vitro* propagation of pomegranate has been reported earlier by Sharon and Sinha (2000) and also Murkute et al. (2004).

In perennial fruit crops, establishment of explants requires special procedures to escape the problem that associated with exudation of polyphenol compounds from cut surface. Different attempts has been made to eliminate browning problem in woody plant species like pre-socking of explants in antioxidants solution, incorporation of oxidants into medium, incubation of culture in to dark period and frequent subculturing of explants (Ahmad et al., 2013). Exudation of phenols can also be reduced by sealing the cut ends of explants with liquid paraffin wax (Bhatt and Chandel, 1991; Singh et al., 2011). However, the effectiveness of these methods varies from species to species and physiological conditions of plant. Corduk and Aki (2011) reported that the addition of 1.0 g/L morpholine ethane sulfonic acid (MES) into MS medium significantly reduced browning in *Sideritis trojana*. Use of antioxidants and absorbents in browning control have been demonstrated by several workers in mango (Chandra et al., 2003), in pomegranate (Chaugule et al., 2007) and in pear (Poudyal et al., 2008). They have also noticed that keeping the culture continuously into dark period for 96 h reduced phenol extraction in pear. Pre-socking of apical and axillary buds in 0.5% polyvinylpyrrolidone (PVP) + 3% sucrose for 30 min was found effective for browning control in mango (Chavan et al., 2000). Production of phenolic compounds indirectly stimulated by various factors such as physiological condition, size and age of explants (Dineshbabu et al., 2002; Tian, 2008; Ahmad et al., 2013).

Therefore, the present investigation was carried out to study the effect of antioxidants, position and size of nodal segment explants on degree of browning and culture establishment of pomegranate cv. Ganesh.

MATERIALS AND METHODS

Explant preparation and surface sterilization

Two weeks old shoots having at least five nodes each were collected from 4 to 5 year old mature plant of pomegranate cv. Ganesh from Horticulture Experimental Farm, Navsari Agricultural University, Navsari, Gujarat. Shoots were washed thoroughly under running tap water for 30 min and leaves were removed leaving the petiole. Sterilization of explants were carried out by keeping in a solution of 0.2% Bavistin (Carbendazim 50% WP) and 0.05% Streptomycin for an hour. Shoots were treated with 10% solution of Teepol for 10 min. All traces of Teepol were removed by repeated washing in double glass distilled water. Pre-sterilized shoots having at least 5 nodes each at different positions (5 levels) viz. 1st, 2nd, 3rd, 4th and 5th from the apex to the base, cut and separated into different size (5 levels) viz. 1.5, 2.0, 2.5, 3.0 and 3.5 cm of each position. Further sterilization procedure was carried out in the laminar air flow hood, using 0.1% mercury chloride ($HgCl_2$) for 5 min. The explants were then rinsed at least thrice with autoclaved double distilled water.

Culture media and culture condition

MS (Murashige and Skoog, 1962) was used as basal medium for the experiment. Analytical grade chemicals, obtained from Hi Media Laboratories (India) were used for media preparation. Screw caps glass bottles (250 ml) were used as culture vessels. The medium was supplemented with 3.0% sucrose and solidified with 0.8% (w/v) agar. The pH of medium was adjusted to 5.8 prior to addition of agar and then medium was autoclaved at 121°C on 15 lb/in^2 for 20 min. Cultures were incubated in a culture room at a temperature of 26 ± 2°C with relative humidity at 55 ± 5% in the 16/8 h light/dark photoperiod at 3000 lux.

Effect of explants position and size

Sterilized nodal segments were inoculated into MS medium fortified with 6-benzylaminopurin (BAP) 1.0 mg/L + Naphthalene acetic acid (NAA) 0.5 mg/L. Total 25 treatment combinations (size of nodal segments 5 levels with each position of node 5 levels) were tested. 2 to 3 explants were inoculated in each 250 ml glass bottles having 40 ml medium. Treatments were replicated three times with 100 explants in each replication. Observations were recorded after one week of culture. Subculturing of explants was conducted at two week intervals.

Effect of antioxidants and subculturing of explants

Effect of antioxidants on browning intensity and frequent subculturing of explants was tested using 2.5 cm nodal segment explants. Different antioxidants viz. activated charcoal (3 levels) 100, 200 and 300 mg/L, polyvinylpyrrolidone (PVP), 3 levels viz. 5, 10 and 15 mg/L, ascorbic acid (3 levels) 50, 100 and 150 mg/L and citric acid (3 levels) 20, 40 and 60 mg/L were added into MS medium with 1.0 mg/L BAP + 0.5 mg/L NAA. Subculturing of explants was conducted at first days after inoculation (DAI), second DAI, first and third DAI.

Statistical analysis

Experiments were carried out using a factorial completely randomized design (CRD). Treatments were repeated at least three times, each treatment consisted of 4 explants and the mean separation was conducted according to least significant differences (LSD) at 5% level. The surface browning of tissue was evaluated visually at every transfer using scores ranging from 1 to 5 (0: no browning, +: very low browning, ++: low browning, +++: moderate browning, ++++: high browning and +++++: intense browning.

Table 1. Effect of position and size of nodal segments explants on browning intensity in pomegranate cv. Ganesh.

Position of node (N)	Size of node (L)				
	1.5 cm	2.0 cm	2.5 cm	3.0 cm	3.5 cm
1st	+	++	+++	+++	+++
2nd	++	+++	+++	++++	++++
3rd	+++	+++	+++	++++	++++
4th	+++	++++	++++	+++++	+++++
5th	+++++	+++++	+++++	+++++	+++++

+++++ = Intense browning, ++++ = High browning, +++ = Moderate browning, ++ = Low browning, + = Very low Brown.

RESULTS AND DISCUSSION

Browning intensity

Minimum browning intensity was observed in the 1st position of nodal explants having 1.5 cm length. The intensity of browning was observed very high in 4th and 5th node. It was greatly increased with increasing the position and size of nodal segments (Table 1). Moreover, the browning intensity in 1st position of node was low to moderate in all the size of nodes. Browning of explants was noticed first at the cut end then gradually diffused into the medium. Exudation of phenols in 4th and 5th position of node was started within a minute of explants inoculated into medium (Figure 1A). It was noticed that the 1st and 2nd nodes having 1.5 to 2.0 cm length showed very low to moderate browning in the medium. However, the establishment percent was also recorded less. Small size explants exudates less phenols (Kaushal et al., 2005) as they are soft and succulent in nature and succumbs easily due to toxic effect of sterilents (Pati et al., 2008). On the other side, 3rd, 4th and 5th position of node showed moderate to intense browning in all (1.5 to 3.5 cm) length of nodes. This might be due to the synthesis of polyphenols more in older age node as compared to new aged node. Older explants exhibited more browning than younger ones (George and Sherrington, 1984). Gitonga et al. (2010) reported low browning intensity in 1st, 2nd and 3rd nodes of macadamia nut shoot. The intensity of browning was correlated with size and position of node. Ozyigit (2008) observed positive relationship between age of explant and phenolic exudation in tissue culture of cotton.

Culture establishment

Maximum establishment (68.5%) was recorded in 3rd nodal explants having 2.5 cm length followed by 1st node with 2.5 cm length (Table 2). Maximum length of shoot (3.9 cm) was induced in 1st position of node followed by in 2nd and 3rd nodes with 2.5 cm length of explants (Table 3). However, minimum establishment was observed in 5th node having 3.5 cm length. Moreover, the establishment of 3rd nodal explants significantly increased due to increase in the length of node up to 2.5 cm. Maldonado et al. (2000) also reported the better culture establishment in 3rd and 4th nodal position of *Annona muricata* L. Pati et al. (2008) observed that the upper node (1st to 5th) did not survive in culture medium, whereas, 11th to 15th nodal segment were found better for establishment in Bael cv. CISHB1. Similarly, Douglas (1984) found that the 4th to 7th internodal explants of papulus species was better for *in vitro* shoot regeneration. Moreover, the increasing trend in establishment of nodal explants was observed up to moderate intensity of browning. Thereafter, the establishment decreased. Decreasing trends in establishment with increasing in size of explants was also reported by Muralikrishna (1988) in pomegranate and Gitonga et al. (2010) in macadamia nut. Establishment and growth of explants was significantly influenced by position and size of nodal explants. Variability in establishment and growth of internodes might be due to the difference in the regeneration potential of different nodes. Regeneration potential of different explants is attributed by the physiological state, age and cellular differentiation among the constituent cells (Murashige, 1974; Laxmi et al., 2013). Moreover, stem internodes contained adequate level of cytokinins for adventitious shoot production (Douglas, 1984). In the present experiment 3rd node having 2.5 cm length was found as best explants for maximum establishment and growth (Figure 1D). This could be due to the less exudation of phenol and endogenous auxin, and cytokinin level in the constituent cells.

Effect of antioxidants and serial subculturing

The data regarding response of antioxidants and frequent subculturing of explants on browning intensity and culture

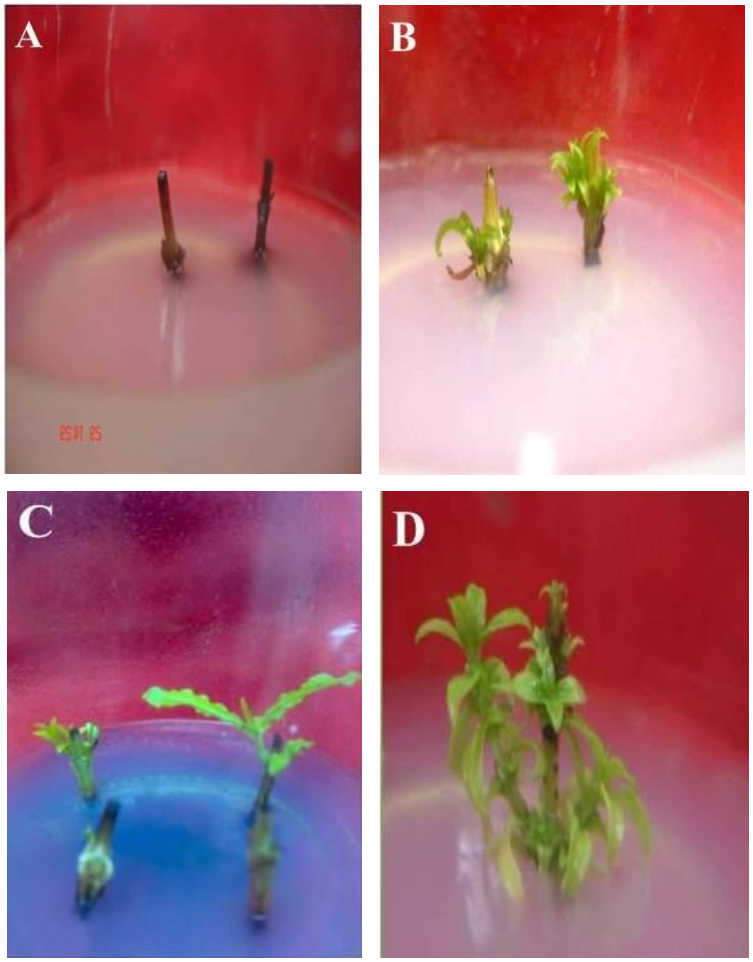

Figure 1. (A) Browning in nodal explants (B Explants after three subcultures at first and third DAI (Days after inoculation) (C) Establishment of 3rd node having 2.5 cm length of explant on 200 mg/l AC (activated charcoal) (D) Growth of 3rd nodal explants having 2.5 cm length after four weeks of culture.

Table 2. Effect of position and size of nodal segment explants on establishment of pomegranate cv. Ganesh.

Position of node (N)	Size of node (L)					Mean (N)
	1.5 cm	2.0 cm	2.5 cm	3.0 cm	3.5 cm	
1st	32.2	39.7	59.1	42.5	37.7	42.24
2nd	27.4	41.3	49.0	37.7	32.2	37.52
3rd	27.6	43.4	68.5	37.5	31.2	41.62
4th	26.5	24.3	25.8	23.7	22.1	24.48
5th	19.11	17.09	16.33	15.04	13.43	16.20
Mean (L)	26.56	33.16	43.75	31.29	27.33	-

S. Em± N = 0.16, L= 0.16, N × L= 0.37. CD at 5% N= 0.47, L= 0.47, N × L= 1.06. N= position of node L= Size of node.

establishment are presented in Table 4. Minimum browning intensity in explant and medium was observed in subculturing treatment at first and third DAI (Figure 1B). Among the different antioxidants, activated charcoal

Table 3. Effect of position and size of nodal segment explants on shoot growth of pomegranate cv. Ganesh.

Position of node (N)	Size of node (L)					Mean (N)
	1.5 cm	2.0 cm	2.5 cm	3.0 cm	3.5 cm	
1st	2.27	2.00	3.97	2.00	1.85	2.42
2nd	1.00	1.96	3.22	2.00	1.87	2.01
3rd	1.75	2.00	3.00	1.25	1.00	1.80
4th	1.65	1.00	0.73	0.84	0.56	0.95
5th	1.07	0.81	0.45	0.39	0.10	0.56
Mean (L)	1.55	1.55	2.27	1.29	1.07	-

S. Em ± N= 0.02, L= 0.02, N × L= 0.05. CD at 5%, N= 0.73, L= 0.73, N × L= 0.16. N = position of node L= Size of node.

Table 4. Effect of antioxidants on *in vitro* degree of browning and culture establishment of pomegranate cv. Ganesh.

Treatments	Browning intensity in medium	Appearance of explants	Cultural establishment (%)
Activated charcoal (mg/L)			
100	++++	Necrotic	12.20 (20.43)*
200	++	Green	41.20 (39.93)
300	+++	Slightly green	19.80 (26.42)
Citric acid (mg/L)			
20	+++	Necrotic	9.60 (18.04)
40	++++	Necrotic	10.20 (18.61)
100	++++	Necrotic	11.40 (19.73)
Ascorbic acid (mg/L)			
50	++++	Necrotic	9.00 (17.45)
100	++++	Necrotic	10.20 (18.62)
150	++++	Necrotic	11.20 (19.54
PVP (mg/L)			
5	++++	Necrotic	9.20 (17.64)
10	++++	Necrotic	10.80 (19.18)
15	+++	Slightly green	11.40 (19.72
Subculturing (DAI)			
One (DAI)	+++	Slightly green	24.40 (29.58)
Two (DAI)	++	Green	37.00 (37.46)
First and third (DAI)	+	Green	60.00 (50.77)
S.Em. ±	-	-	0.35
CD at 5%	-	-	1.01

*Figure in parentheses are arcsine transformed value. Browning intensity - ++++ Intense browning, ++++ High browning, +++ Moderate Browning, ++Low browning, + Very low Brown.

200 mg/L was found better in reducing of medium and explants browning (Figure 1C). However, addition of 300 mg/L activated charcoal into medium adversely affected culture establishment and shoot growth. Citric acid and

ascorbic acid did not show any effect in browning control. Whereas, PVP 15 mg/L reduced explants browning to some extent. Maximum culture establishment (60.0%) was recorded in frequent subculturing of explants at first and third DAI followed by in 200 mg/L activated charcoal (41.2%). Similarly, the appearance of the explants was green in all the subculturing treatments. The results are coincident with the findings of Murkute et al. (2004) and Singh and Khawale (2006).

The presence of phenolic compounds in explant tissues is a serious problem for *in vitro* culture establishment (Compton and Preece, 1986). These phenolic substances exudate from the cut surface of explants and oxidized due to the preoxideses, polyphenols or air (Onuoha et al., 2011) resulting in the medium turning brown and death of the explants (Aliyu, 2005). Addition of the antioxidants into culture medium is quite effective for controlling medium browning, as it removes the quinines formed in the medium.

Several studies have reported the use of antioxidants in browning control in perennial fruit plants (Khattak et al., 1994; Vasar et al., 2003; Birmeta and Welander, 2004; Zamir et al., 2004; Patil et al., 2011). Whereas, in the present study, ascorbic acid and citric acid was ineffective in control of browning. In contrast with our results, Patil et al. (2011) found best results in browning control with 150 mg/L ascorbic acid and 100 mg/L citric acid in pomegranate. Similarly, PVP was also found less effective in browning control. Tyagi et al. (1981) and Prajapati et al. (2003) effectively controlled explant browning with PVP when added into medium. The effectiveness of different antioxidants in control of browning is varying among plants and species. This could be due to the specificity of these chemicals to certain plant and species. The specificity of PVP in browning control was also reported by Vaugh and Duke (1984). Further, addition of activated charcoal 300 mg/L reduced the growth of explants. It might be due to the absorption of nutrients from medium. Activated charcoal is a strong phenol adsorbent (Zhou et al., 2010) that reduces phenolic browning in explants. It absorbs not only toxic substances and phenols (Fernando et al., 2010) but also the higher amount of growth regulators and nutrients in medium. The most effective browning control measure was subculturing of explants twice, at first day and third day of inoculation of explants. Frequent transfer of explants within the same medium or into fresh medium fairly prevents *in vitro* browning of explants (Kotomory and Murashige, 1965; Block and Lankes, 1996).

Frequent transfer of explants into fresh medium seals cut end of the explants that stopped leaching of phenols (Ahmad et al., 2013). These results are in parallel to those of Muralikrishna (1988), Singh and Khawale (2006) and Singh et al. (2011). They claimed that the subsequent transfer of explants on fresh medium resulted in complete disappearance of browning in nodal segment explants of mature plants in pomegranate.

Conclusion

Position of node in the shoots of mother plant and node size has great influences on the *in vitro* degree of browning. Among all the node positions, 3rd node with 1.5 to 2.5 cm length showed higher establishment and growth of explants with less browning intensity. Furthermore, the most effective browning control measure was subculturing of explants twice, first and third day of inoculation. Addition of 200 mg/L activated charcoal into the medium was found quite effective to minimize browning problem in nodal segment of mature explants.

Conflict of Interests

The authors have not declared any conflict of interest.

Abbreviations

MS, Murashige and Skoog medium; **NAA,** naphthalene acetic acid; **BAP,** 6-benzylaminopurine; **AC** activated charcoal; **PVP,** polyvinylpyrrolidone; **DAI,** days after inoculation.

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Full Length Research Paper

Seed size polymorphism in *Khaya senegalensis* (Desr.) A. Juss.: Implications for seed propagation

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Seed size variation has implications for the success of seedling establishment, but the underlying mechanisms are yet to be fully explored in many species, including *Khaya senegalensis*. Moreover, seed size is measured in different ways (for example, mass or length), but the extent to which these different ways of measurement differ in predicting seedling growth parameters is unknown. In this study, how well seed mass and seed length predict seed food reserves was tested. Then, pot experiments were conducted to determine which of the two measures of seed size was a better predictor of seedling size and root biomass allocation. Also, effects of seed size variation and its relation to sowing depth on seedling parameters were investigated. Results showed that both seed mass and seed length significantly predicted the amount of seed food reserves, but seed mass explained a greater percentage of the variability in seed reserves than seed length (64.1% versus 19.3%) and as a result, seed mass also better predicted seedling size. However, both seed mass and seed length poorly predicted root length and root biomass allocation. Also, it was found that at all the tested sowing depths in this study, larger seeds produced larger and taller seedlings, but a combination of large seeds with 0 cm sowing depth yielded the largest and tallest seedlings. Root length decreased with sowing depth, regardless of seed size. Root mass fraction of seedlings from small seeds decreased with sowing depth, while those from large seeds were unaffected. It is recommended that to produce larger seedlings with a greater allocation to root biomass, large seeds in combination with superficial sowing depth should be used when nursing *K. senegalensis* seeds.

Key words: Seed size variation, sowing depth, seedling size, root biomass, *Khaya senegalensis.*

INTRODUCTION

Seed polymorphism is defined as “the production of two or more distinctly different types of seeds by a species” (Harper et al., 1970). Seed size polymorphism therefore refers to size variations in seeds produced by a species. A sizeable body of knowledge exists on this phenomenon (Poulin and Hamilton, 2000; Simons and Johnston, 2000; Einum and Fleming, 2002). In many species, seed size variation has important connection to the overall

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biological fitness of parental species, by directly affecting the process of germination, seedling recruitment and competitive ability (Shaukat et al., 1999; Leishman et al., 2000; Coomes and Grubb, 2003; Souza and Fagundes, 2014).

According to Leishman et al. (2000), "seed size of a species represents the amount of maternal investment in an individual offspring, or how much 'packed lunch' an embryo is provided with to start its journey in life". But which is a better way to measure "packed lunch"; mass or length of 'lunch box'? This question represents an important challenge at the nursery when raising seedlings from species that vary in seed size.

Khaya senegalensis (Desr.) A. Juss., belonging to the family Meliaceae is a savanna tree species of enormous socio-economic importance, but has poor natural regeneration (Nikiema and Pasternak, 2008). Plantation development of this species is necessary for perpetual flow of benefits. It is recognized that production of good quality planting stock is a critical first step to successful plantation establishment. However, apart from variations in seed size among individuals within species resulting from differences in environment, seed size of *K. senegalensis* is also known to vary greatly among provenances (Ky-Dembele et al., 2014). In this species, seeds could be easily grouped into different size classes based only on ocular estimates of seed length. Because seeds are winged as an adaptation to dispersal, variations in wing size may imply that for some seeds, not the entire length of the seed is filled with the endosperm. Also, variation in seed thickness makes it much more likely that great variability in seed mass may exist even among individuals that appear to have the same length. Therefore, knowledge of relationships between the various ways of measuring seed size (and between them and cotyledon mass, which is a measure of seed food reserves) is needed to be able to make right choices at the nursery. However, such data is lacking, particularly for this species.

Additionally, larger seedlings are required for higher establishment success in the savanna due to the frequent bush fires and the longer dry seasons in this environment (Fensham et al., 2003). This is important because planted seedlings of *K. senegalensis* are fairly susceptible to fires (Orwa et al., 2009) and are also known to suffer dry season drought stress (Arndt, 2015). Larger seedlings survive better because they have higher carbohydrate reserves (Westoby et al., 1996; Leishman et al., 2000), but the amount of carbohydrate reserves correlates with root mass fraction (RMF) and both traits are known to enhance drought survival (O'Brien et al., 2010) and post-fire re-sprout capacity (Hoffmann et al., 2004) among seedlings of savanna species. Therefore, to achieve higher seedling establishment success under harsh environmental conditions, larger seedlings or seedlings with a higher allocation to root biomass are needed. This may be accomplished by picking out and sowing large seeds and at the right sowing depths. Sowing depth is important because seeds sown deeper take a longer time to emerge, requiring much more energy to be expended. This could affect seedling size and competitive ability (Tripathi and Bajpai, 1985). Also, in containerized planting, deep sowing could obstruct root development.

Data on effect of seed size variation on seedling traits in this species are scarce (Ky-Dembele et al., 2014), but even more scarce are studies that have explored the interaction effects of seed size and sowing depth on seedling size and root biomass allocation. In this paper, findings on experiments in which the extent of the relationship of seed food reserves to seed mass and seed length are presented, and also, which measure of seed size better predicts seedling size and root biomass allocation was determined. The main and interaction effects of variations in seed size and sowing depth on seedling size and root biomass allocation was also determined.

MATERIALS AND METHODS

Study site

The experiments were carried out at the plant house of the Nyankpala Campus of the University for Development Studies, Tamale. The site is located within the Guinea savanna ecological zone in the Tolon district of Northern Region of Ghana. Geographically, the district lies between latitude 9° 25'N and longitude 0° 58'W. Average mid-day temperature at the plant house for the month of March, 2015 (when the experiments were conducted) was 29°C. The roof of the plant house reduces irradiance level by up to 40%.

Seed collection and study approach

In February, 2015, seeds of *K. senegalensis* were collected under 40 fruiting trees within the Tamale Metropolis located in the Guinea savanna ecological zone in Northern Ghana. Seeds gathered were put together in a 25 m^3 sack. The seeds were used in two separate experiments. The first experiment was conducted to determine the extent of the relationships of seed mass and seed length to seedling size and root biomass allocation. In this experiments, the extent of relationships of seed mass and seed length to cotyledon dry mass (a measure of seed food reserves) was also quantified with a view to establishing which of the two (that is, mass or length) better predicts amount of seed reserves in *K. senegalensis*. The second experiment was carried out to establish main and interaction effects of seed mass with sowing depth on seedling size and root biomass allocation of seedlings. Prior to conducting the plant house experiments, some seeds were sampled for determination of seed reserves.

Determination of seed reserves

A total of 500 seeds were picked at random from a large seed pool. Fresh mass (g) and length (cm) were measured of each seed using an electronic scale and a ruler, respectively. Samples were then oven-dried at 70°C for 48 h after which seed coats were removed. The endosperms (cotyledons) were weighed to obtain cotyledon dry mass (which was used as a measure of seed food reserves).

Experiment I

Design, layout and data collection

Another 500 seeds were sampled and weighed. With the help of a divider and a ruler, lengths (cm) of the fresh seeds were taken along the long axis of each seed, making sure only cotyledon (endosperm) length was obtained. This was necessary because seeds of this species are winged. Seeds were then sown in rectangular seed boxes (with dimensions 50 cm × 15 cm × 10 cm) at 2.5 cm depth. At the start of the experiment, each seed box received 1000 ml of water per day given in a twice daily dose (morning and evening). This quantity was reduced to 500 ml after 2 days to avoid soil saturation. Emergence started 5 days after sowing and amount of water given was again increased to 1000 ml per day to cater for the increasing water demand. The position of each seed was marked. This was crucial because although each box contained 20 seeds, each seed was an experimental unit. Boxes only served as seed beds.

The number of days it took for each seed sample to emerge was recorded, noting samples that failed to emerge at the end of the experiment (that is, 90 days after planting). Seedling height of all samples was measured. They were then uprooted, tagged and oven-dried at 70°C for 48 h, and separated into root, stem and leaves and each part weighed separately. Taproot length was measured prior to oven-drying. Total seedling dry mass was calculated by summing up root, stem and leaf dry mass. Root mass fraction (RMF) was then determined by dividing root dry mass by total seedling dry mass.

Experiment II

Design, layout and data collection

For this experiment, 480 seeds were picked at random from the seed pool. Seed fresh mass was determined following same protocol as in experiment I. Seeds were then put into one of two size categories; large seeds (> 0.35 g) and small seeds (< 0.25 g).Seeds were sown in seed boxes (same dimensions as those used in experiment I) at three different depths; 0, 2.5 and 5.5 cm. Zero cm sowing depth meant placing the seed on the soil surface without covering with soil. Each size-depth treatment combination (total of 6) was assigned to a seed box in a completely randomized design (CRD) such that each box represented an experimental unit. Each treatment was replicated 4 times. Twenty seeds were sown in each box. Soils for this experiment were taken from top 10 cm in a mango plantation of the Faculty of Renewable Natural Resources, Nyankpala. No fertilizers were added. Watering regime was same as in experiment I. The experiment ended 65 days after sowing. 7 seedlings were randomly sampled from each box and height of each sample was measured with a ruler. The samples were uprooted and their taproot lengths were measured. They were then separated into roots, stems and leaves and oven-dried at 70°C for 48 h. Dry weight of roots, stems and leaves were measured with an electronic scale. Total seedling dry mass and root mass fraction were determined following same protocol as in experiment I.

Data analysis

Data from the 500 seeds used for the determination of seed reserves were combined with the 500 seeds from experiment I and explored for descriptive statistics (n = 1000 seeds). Means and standard deviations were then used to determine coefficients of variation for seed mass and seed length. To determine the better predictor of seed food reserves, separate linear regression analyses were conducted with seed mass and seed length as predictors and cotyledon dry mass as the dependent variable. Also the measure of seed size which better predicts seedling size and root biomass allocation was determined by subjecting each seedling trait measured in experiment I to linear regression analysis. Pearson's correlation coefficient was used as a measure of strength of the relationships between each seedling trait and the predictor (that is, either seed mass or seed length). Two regression equations, one for each predictor, were also derived for each measured seedling parameter.

To determine main and interaction effects of seed size and sowing depth on seedling size and root biomass allocation, a multivariate analysis of variance (MANOVA) was performed on seedling height, seedling total dry matter and RMF. Our choice test statistic was Roy's Largest Root as that proves more powerful with smaller sample sizes (Olson, 1974, cited in Field, 2009). A MANOVA was chosen over multiple ANOVAs due to the possibility of relationships existing among the dependent variables (that is, seedling dry mass, seedling height, root length and RMF), but more importantly to control familywise error rates (Field, 2009). Where a significant interaction effect of seed size and sowing depth was found, adjustment for multiple comparisons was done using SIDAK. All analyses were done on SPSS version 22.0 (IBM Corp., 2013).

RESULTS

Is there evidence for seed size variation?

Seed mass varied from 0.1 to 2.8 g (range = 2.70 g). The mean seed mass was 1.117 ± 0.026 g. The coefficient of variation for seed mass was 76.4%. Seed length varied from 0.15 to 2.8 cm (range = 2.65 cm). Mean seed length was 1.156 ± 0.0584 cm. The coefficient of variation for seed length was also found to be very high (75.8%).

Which predicts seed food reserves better: seed mass or seed length?

Seed mass and seed length were significantly correlated ($r = 0.482$, $p < 0.001$). Therefore, both produced regression models that significantly ($F_1 = 888.499$, $p < 0.001$ and $F_1 = 120.500$, $p < 0.001$, respectively) predicted cotyledon dry mass (seed food reserves). However, the amount of variation in cotyledon dry mass explained by seed mass was higher (64.1%) than variation explained by seed length (19.3%) (Figure 1A and B). The resulting regression equations are $Y = 0.28 + 0.613X$ and $Y = 0.070 + 0.02X$ for seed mass and seed length, respectively.

Which better predicts seedling size and root biomass allocation; seed mass or seed length?

Results of the linear regressions conducted on data from experiment I revealed significant correlations between seed mass and seedling dry mass (Figure 2A), and between seed mass and seedling height (Figure 2C). However, correlations between seed mass and both root length and RMF were not significant (Figure 2E and G,

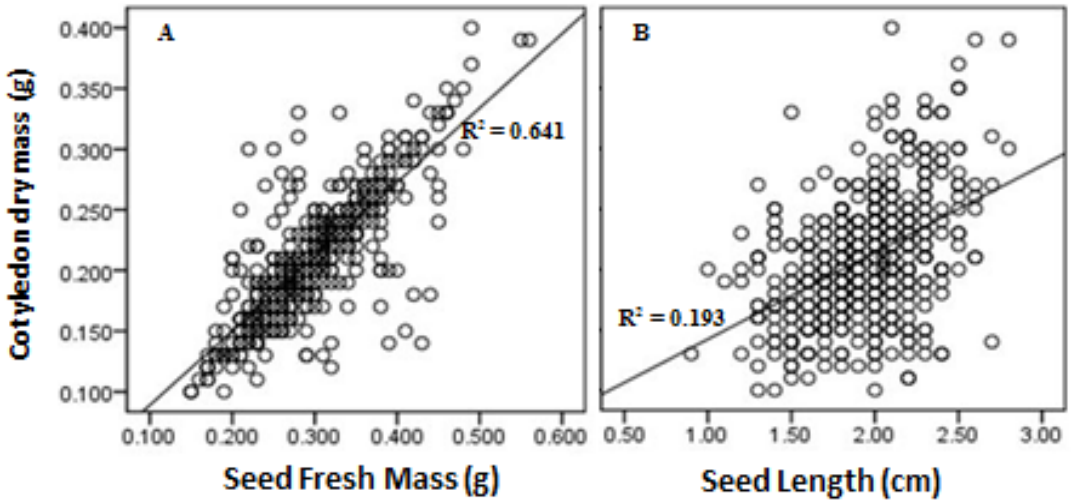

Figure 1. Relationships of cotyledon dry mass (seed reserves) with seed mass (A) and seed length (B). (N = 500 seeds).

Table 1. Regression equations for measured seedling parameters with seed mass and seed length used as predictors in the model.

Parameter	Regression equation	
	Seed mass (g)	Seed length (cm)
Plant dry weight (g)	Y = 0.228 + 0.620X (**)	Y = 0.377 + 0.016X (ns)
Seedling height (cm)	Y = 13.305 + 7.076X (*)	Y = 11.94 + 1.704X (*)
Root length (cm)	Y = 7.069 + 0.752X (ns)	Y = 7.115 + 0.086X (ns)
RMF (gg^{-1})	Y = 0.150 + -0.009X (ns)	Y = 0.097 + 0.025X (ns)

** $p < 0.001$; * $p \leq 0.05 > 0.001$; ns = no significant difference.

respectively). Seed length on the other hand, correlated significantly with seedling height and RMF (Figure 2D and 2H, respectively), but did not correlate significantly with seedling dry mass and root length (Figure 2B and 2F, respectively). Seed mass as a predictor produced a regression model that predicted seedling dry mass and height significantly (F_1 = 12.710, p < 0.001 and F_1 = 5.046, p = 0.025, respectively), but did not significantly (F_1 = 0.246, p = 0.620 and F_1 = 0.019, p = 0.889, respectively) predict root length and RMF. Seed length on the other hand produced a regression model that significantly (F_1 = 5.946, p = 0.015) predicted seedling height, but regression models for seedling dry mass, root length and RMF were not significantly (F_1 = 0.177, p = 0.674; F_1 = 0.066, p = 0.798 and F_1 = 3.233, p = 0.073, respectively) predicted by seed length. The resulting regression equations are presented in Table 1.

What are the effects of seed size and sowing depth on seedling size and root biomass allocation?

Results of the MANOVA revealed significant (V = 1.247, $F_{4, 16}$ = 4.987, p = 0.008, η^2 = 0.55) interaction effects of sowing depth and seed size on mean seedling height, total dry mass, root length and RMF. Separate univariate ANOVAs on the outcome variables revealed significant main effects of seed size (F_1 = 47.99, p < 0.001, η^2 = 0.727) and sowing depth (F_2 = 9.355, p = 0.002, η^2 = 0.51) on seedling dry mass. The general pattern revealed was that regardless of seed size, seedling dry mass decreased with sowing depth and large seeds consistently produced larger seedlings regardless of planting depth (thus, no interaction effect of seed size × sowing depth was detected with the F-test). However, pairwise comparisons (with SIDAK adjustment) showed that a combination of large seeds with 0 cm sowing depth yielded the highest dry matter (Figure 3A). Just as in the case of seedling dry mass, it was found that although the F-test did not produce a significant (F_1 = 2.835, p = 0.08) seed size × sowing depth interaction effect on seedling height, pairwise comparisons showed that large seeds were significantly (p = 0.02) taller than those from small seeds at 0 cm sowing depth, but this effect of seed size was not found at higher sowing depths (Figure 3B).

Root mass fraction (RMF) was significantly (F_2 = 6.27,

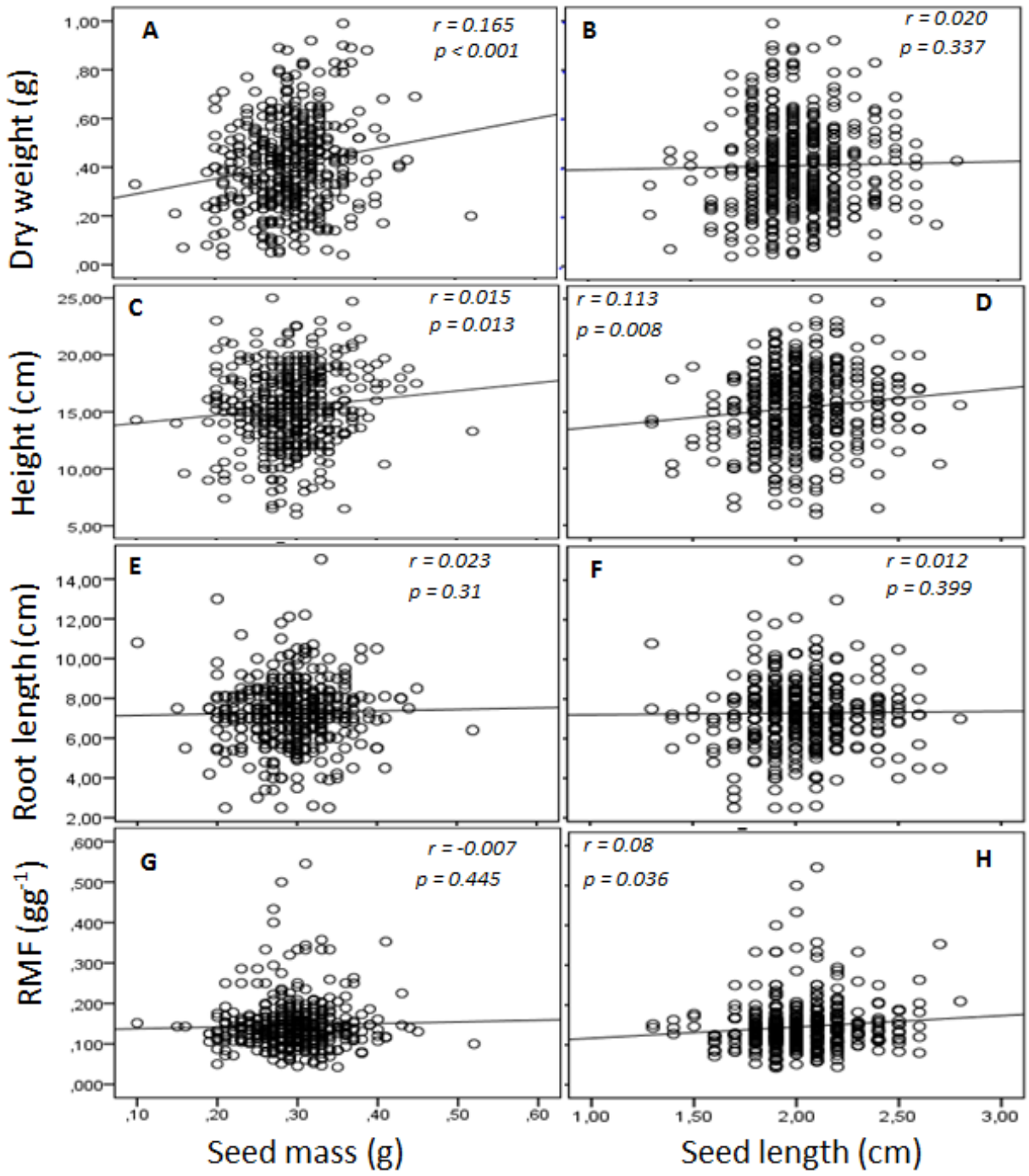

Figure 2. Relationships of measured seedling parameters to seed mass and seed length. N = 500 seeds. The extent of correlations between seed and seedling parameters is shown with r and its p value.

p = 0.009, η^2 = 0.41) affected by the interaction of seed size and planting depth. Pairwise comparisons revealed that whereas seedlings from large seeds were not significantly affected by planting depth, seedlings from small seeds had lower RMF at higher sowing depths, such that the two seed size categories differed significantly at 5.5 cm sowing depth. For small seeds, RMF at 0 and 2.5 cm sowing depths were similar but both were significantly (p = 0.001 and p = 0.002, respectively) higher than 5.5 cm sowing depth (Figure 3C). Root length was significantly (F_2 = 22.797, p = < 0.001, η^2 = 0.717) affected by sowing depth. The effect size of sowing depth on root length was very high. Seed size effect on root length was not significant (F_1 = 1.278, p = 0.27, η^2 =

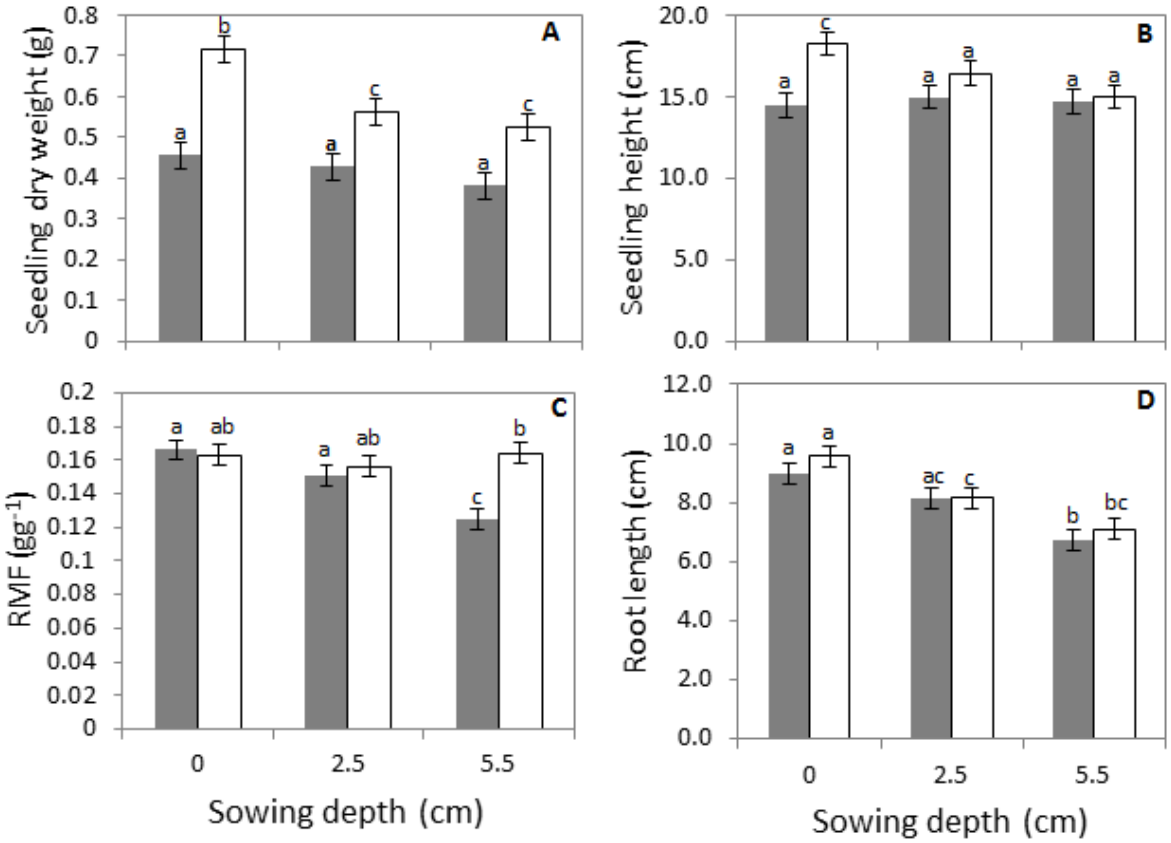

Figure 3. Estimated marginal means of (A) seedling dry matter, (B) seedling height, (C) RMF and (D) root length of the two seed size classes at different sowing depths. Open bars represent seedlings from large seeds and grey-filled bars are seedlings from small seeds. Letters indicate significant (different letters) or non-significant (same letters) differences at 0.05 level of significance. Error bars are standard errors from table of estimated marginal means after pairwise comparisons. Adjustment for multiple comparisons was done using SIDAK.

0.06). Pairwise comparisons revealed that root length was highest at 0 cm sowing depth and lowest at 5.5 cm sowing regardless of seed size (Figure 3D).

DISCUSSION

Plantation development is increasingly becoming relevant as natural forests begin to succumb to anthropogenic pressure. For many species, raising good quality planting stock from seeds is a critical first step. Seed size is clearly important, but important questions remain unanswered about the extent of seed size variation and its exact effects in many species. These questions were investigated in *K. senegalensis* and a very high size variability among seeds was found. This was the case whether seed size was measured either in mass or in length. Due to both genetic variability and differences in site resources and/or conditions, individuals of the same species could vary greatly in sizes of seeds produced (Leishman et al., 2000; Halpern, 2005). Seeds used in our experiments came from many individuals which may also belong to different provenances. This may explain the high variability in seed size observed in this study. This does not represent a limitation in methodology because seeds used in large scale nursery operations are often collected from many individual trees. Moreover, between-provenance variability in both seed length and seed mass has already been demonstrated in this species (Ky- Dembele et al., 2014).

Also, it was found that both seed length and seed mass significantly predicted seed reserves, but seed mass was a better predictor than seed length because it explained a greater percentage of the variability in seed reserves than seed length. Thus, there were many seeds of same length that had different amounts of seed reserves than there were seeds of same mass that had varying amounts of seed reserves. Therefore, seed mass also predicted seedling height and dry matter yield better than seed length, although both did not predict root length and root biomass allocation very well. These findings are consistent with the expectation as it is known in many species that the amount of seed reserves determines seedling size (Westoby et al., 1996; Leishman et al.,

2000). The implication of this finding is that it is better for seeds to be selected for sowing based on seed mass rather than seed length, in spite of the fact that it may be easier to pick out seeds based on length as length appears to be more easily estimated by ocular means than seed length, which has to be measured. This is important because the amount of seed reserves determines success of planted seedlings via its influences on seedling size (Westoby et al., 1996; Coomes and Grubb, 2003).

The second experiment revealed that larger seeds produced larger and taller seedlings than smaller ones, but there was a decreasing pattern of seedling size and height with sowing depth such that differences in height between the two seed size categories existed at the highest sowing depth (5.5 cm). This may be because more reserves (energy) was needed to emerge from deeper layers, consistent with findings in other species (Tripathi and Bajpai, 1985; Schmidt, 2000). Additionally, it was found that both RMF and root length did not depend on seed size, but both decreased with sowing depth, possibly due to physical limitation of container. It was also observed that RMF of seedlings from smaller seeds suffered the adverse effect of deep sowing, but seedlings from larger seeds were not affected. The deeper a seed is sown in a container the less space the roots have to extend into deeper layers because of the physical limitation imposed by the bottom of the container.

Conclusion

Seed size variation has important implications for seedling success. The study investigated the extent of this phenomenon in *K. senegalensis* and explored its influences and underlying mechanism on seedling traits that are crucial for field survival. Seeds of this species vary greatly in terms of both length and mass, but it is better to measure size as mass rather than length of seed, as mass predicts seed reserves better than length.

Also, findings revealed that the size of sown seed determines seedling dry mass and height, with larger seeds producing larger and taller seedlings than smaller ones. However, while seed size does not determine how much biomass is allocated to roots or how deeply rooted the seedlings are, sowing depth determines both the size of the seedling obtained and how deeply rooted the seedlings are in the container. The deeper the sowing depth, the shorter the roots of resulting seedlings. Root mass fraction also decreases with sowing depth when seedlings are small.

It is suggested that a combination of large seedlings with shallow sowing depth (shown in this study as > 0.35 g and 0 cm, respectively) yields the largest seedlings with the highest allocation to root biomass and therefore recommended for use when raising *K. senegalensis* seeds in containers.

Conflict of interest

Authors have not declared any conflict of interest.

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