ANATOMICAL, GERMINATION AND *IN VITRO* STUDIES ON SHEA TREE (*Vitellaria paradoxa* C.F.Gaertn.) SEED

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DECLARATION

‘‘This thesis is the result of research work undertaken by Iddrisu Abdulai in the Department of Nuclear Agriculture and Radiation Processing of the School of Nuclear and Allied Sciences, University of Ghana, under the supervision of Prof. George Y. P. Klu and Dr. Kenneth E. Danso.’’

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DEDICATION

This work is dedicated to the Almighty Allah for giving me knowledge, wisdom and all other resources to complete it successfully, to my late friend Nategu Naah Mahama for his effort at collecting shea fruits for this work and to Franklin Otsyina for his support and encouragement.

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of variance
BA	-	6-benzyladenine
BAP	-	6-Benzylaminopurine
CBE	-	Cocoa Butter Equivalent
CER	-	Cryptocotylar epigeal reserve
CHR	-	Cryptocotylar hypogeal reserve
CRIG	-	Cocoa Research Institute of Ghana
$CuSO_4$	-	Copper sulphate
DAS	-	Days after sowing
DE	-	Distal end
DS	-	Dorsal side
EI	-	Emergence index
EP	-	Emergence percentage
ERI	-	Emergence rate index
EST	-	Establishment
IPGRI	-	International Plant Genetic Resources Institute
GAEC	-	Ghana Atomic Energy Commission
GP	-	Germination percentage
GS	-	Germinated seeds
$HgCl_2$	-	Mercuric(II) chloride
HTML	-	HyperText Markup Language
LA	-	Leaf area
LW	-	Length and width
LSD	-	Least significant difference
MGT	-	Mean germination time
MS	-	Murashige and Skoog

N	-	North
NAA	-	Naphthaleneacetic acid
PE	-	Proximal end
PEF	-	Phanerocotylar epigeal foliaceous
PER	-	Phanerocotylar epigeal reserve
PHF	-	Phanerocotylar hypogeal foliaceous
PHR	-	Phanerocotylar hypogeal reserve
PRE	-	Pseudoradicle elongation
psi	-	pound per square inch
SA	-	Shoot appearance
SAM	-	Shoot apical meristem
SE	-	Shoot elongation
SSA	-	sub-Saharan Africa
TS	-	Total number of sown seeds
TTC	-	Tetrazolium chloride
TTZ	-	Topographical tetrazolium
UV	-	Ultraviolet
VS	-	Ventral side
2,4-D	-	Dichlorophenoxyacetic acid

ABSTRACT

In vivo and *in vitro* germination and regeneration studies were conducted on the development of *Vitellaria paradoxa* seedlings as an initial effort towards its domestication. However, to achieve this objective, the morphology and anatomy of the seeds were first studied because they influence germination. Although a smooth, brown coat encloses a *V. paradoxa* seed, it did not impose dormancy on the embryo. Transverse and longitudinal sections through the seed showed that the embryo is surrounded by latex- and fat-containing tissues which made its identification difficult. Thus, the embryo was identified by immersing transversely cut seeds in 1.0 % tetrazolium chloride (TTC) solution for 24 hours which stained it red. When *V. paradoxa* seeds of similar size were sown on nursery beds, the resulting seedlings developed through seven stages namely sprouting, pseudoradicle elongation, bulging, appearance of the shoot on the pseudoradicle, shoot elongation, emergence and seedling establishment. The pseudoradicle is the fused petioles of the two cotyledons and a transverse section through it revealed an outer sheath and lactiferous vessels surrounding a central hollow tube. Longitudinal section also showed the lactiferous vessels surrounding the central hollow tube in which the plumule moves through until it reaches the bulge of the pseudoradicle where it develops into a rudimentary shoot. The rudimentary shoot then protrudes from the pseudoradicle and grows upwards. Classifying seeds into three groups based on sizes and sowing them on nursery beds showed that seed size significantly affected days to germination and the morphology of the resulting seedlings. Large seeds germinated within one week after sowing with vigorous growth compared to small and medium seeds. Although the seedcoat of *V. paradoxa* never imposed dormancy, deshelling (removal of the seedcoat) significantly led to early germination and synchronous seedling emergence compared to those for

intact seeds (control). *In vitro* culture of intact and deshelled seeds on Murashige and Skoog (1962) basal salts modified with 6-benzylaminopurine (BAP) produced no plantlets although 80 % of the deshelled seeds developed long pseudoradicles on a medium supplemented with lower concentration of BAP (1.0 or 2.0 mg/l). Similarly, the culture of TTC identified embryonic axes did not produce plantlets, but rather significantly long pseudoradicles were produced with BAP having significant effect on pseudoradicle development. Contrastingly, *in vitro* culture of excised rudimentary shoots on the same MS medium modified with BAP and naphthaleneacetic acid (NAA) produced plantlets with distinct shoots and leaves. Significant reduction in days to emergence of seedlings from deshelled seeds and successful *in vitro* plantlet development using rudimentary shoot explants will enhance nursery establishment of this economically important tree species for domestication and reafforestation programmes in sub-Saharan Africa.

CHAPTER 1

1.0. Introduction

The shea tree (*Vitellaria paradoxa* C.F.Gaertn.) belongs to the family Sapotaceae. It is indigenous to sub-Saharan Africa (SSA) and typically occurs in the interior savannas where it is the major oil crop (Nikiema and Umali, 2007). The plant still remains an indigen confined to its native 19 countries located in SSA. In Ghana, shea trees are commonly found in the northern sector with sparse populations in Brong-Ahafo, Ashanti, Eastern and Volta regions of southern Ghana (Fobil, 2007).

Now, the importance of shea tree to the local inhabitants of the Shea Belt chiefly depends on the time when its products (shea fruit, nut and butter) are available, late March to September. The early part of this period represents the time when energy demands for farm labour are highest, whilst food supply is at its lowest. Hence, the shea fruit is most frequently consumed as a staple food. The fruit pulp is nutritious containing large quantities of vitamin C, proteins and minerals (Ugese *et al*., 2008a,b; Maranz *et al*., 2004). In rural areas, the nuts are either bartered for starchy foodstuffs or sold immediately and the money used to buy them (Yidana, 2004). The product of international trade which is extracted from the nuts is shea butter.

Shea butter has characteristics similar to those of cocoa butter; hence, it is used as a cocoa butter equivalent (CBE) to manufacture confectionery (Shea Matters, 2011). It is also used as a base for medicines and lotions in the pharmaceutical and cosmetic industries respectively. The fat is used locally as cooking oil, for soap making and as fuel for lighting lamps. Medicinally, almost every part of the tree is used, including the epiphytes *Tapinanthus* spp. which normally parasitize the species. The protein-

rich defoliatory caterpillar *Cirina butyrospermi*, associated with this species, is widely consumed in Nigeria and other parts of the Shea Belt (Ande, 2004).

Vitellaria paradoxa is an iconic and unique tree of the Sudano-Sahelian savanna landscape. Nutritionally, shea fruits are available during the lean season (Sanou and Lamien, 2011). Morphoagronomically, shea trees grow abundantly on marginal soils and have high longevity. Ecologically, they proliferate in arid and semiarid savannas and this proliferation manifests the species' capabilities to combat desertification, to ameliorate the microclimate and to recycle nutrients through annual leaf shedding (Dianda *et al.*, 2009). Socioeconomically, shea provides employment and income to women and children who are the most vulnerable in society (Elias and Carney, 2007). With both the kernels and butter as export commodities, *V. paradoxa* thus has the potential of bridging the economic gap between Northern and Southern Ghana.

Despite all these benefits and potentials, *V. paradoxa* still remains wild (Nyarko *et al*., 2012; Okao *et al*., 2012) and lacks a tradition of being planted (Sanou and Lamien, 2011). Traditionally, farmers rarely plant shea tree because the seedlings grow extremely slowly (Ugese *et al.*, 2010a) leading to the long gestation period of about 15–20 years (Masters *et al.*, 2004). Seasonally, shea trees bear fruits erratically and this unpredictable yielding pattern negatively affects the agro-industrial development of the species and the food security of rural dwellers whose livelihood depends on it (Yidana, 2004). Prices of shea products are also disappointingly low.

Of all the factors, the long juvenile growth period of the tree is main disincentive to the food-insecure farmers of the Shea Belt and has been primarily blamed for the non-

domestication of the plant (Shea Matters, 2011; Moore, 2008). Thus, a substation was established by Cocoa Research Institute of Ghana (CRIG) at Bole in 1976 to research into the ecology of *V. paradoxa* and to develop early-bearing varieties and techniques for propagating them. In spite of CRIG's efforts which resulted in some successes on vegetative propagation, no major breakthrough in the reduction of the gestation period has been reported (Yeboah *et al*., 2011). Consequently, the tree still remains wild.

Wild *Vitellaria* trees now face more threats than ever, with natural stands being extensively cleared for establishing other high-income earning cash crops such as mango (*Mangifera indica* L.) and for producing fuelwood and charcoal. Osei Agyeman *et al*. (2012) reported that mature shea trees are the most preferred woody species for charcoal burning in the Upper West Region of Ghana with an average of 4 trees felled per month per charcoal burner. As a result, the Sudan savanna zone which in the 1940s had the densest population (230 trees ha^{-1}) now has as few as 5 trees ha^{-1} (Djossa *et al*., 2008). Thus, developing efficient propagation techniques to produce seedlings or plantlets for planting, or reafforestation is highly recommended.

Prospects and evidences of crop improvement by intensively growing the genetically unmodified *V. paradoxa* seedlings abound. Yidana (2004) reported that trees on agroforestry parklands produce higher yields and bear fruit more consistently than those in bushlands. Also, fruit yield of individual trees vary extensively, with some trees even bearing fruits bi-annually. These characteristics of *V. paradoxa* trees suggest that selection for yield improvement is a real possibility (Nyarko *et al*., 2012). Therefore, higher yields are obtainable when seedlings or plantlets produced directly from plus trees are purposefully planted to establish plantations.

Intentionally planting *Vitellaria* seedlings to establish plantations should be considered as a viable initial effort towards domesticating this species because the shea industry still relies on fruits collected from the wild. The major challenge to this approach of establishing shea plantations is how to produce uniform and vigorous seedlings in large quantities. First, the seed is recalcitrant and thus germinates readily after harvest but loses viability rapidly (Orwa *et al*., 2009). Second, seedlings produce long taproots which adapt them well to their savanna habitat (Jackson, 1974), but the long taproots make raising them in the nursery and subsequent transplanting difficult. Therefore, germination studies are necessary to circumvent some of these problems, especially by developing seedlings with shorter taproots.

In vitro propagation techniques may offer a feasible alternative for producing planting materials abundantly. However, all the vegetative parts of *V. paradoxa* including shoot tips which are possible explants exude latex copiously. Latex hinders vegetative propagation (Masters, 2002), often contaminating *in vitro* plant cultures massively. Latex-related contamination may be minimized when explants with limited amount of latex are identified and excised for culture. For example, the immature cotyledons and the pink-coloured juvenile shoot located in the bulged portion of the pseudoradicle of the germinated seeds may contain little or no latex. The immature cotyledon explants were cultured on Murashige and Skoog (1962) basal medium supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) and somatic embryos were induced and successfully transformed into embryogenic calli (CRIG, 2012).

Although Ugese *et al*. (2010a) and Jackson (1968) have already described the type of germination and the stages associated with *Vitellaria* seedling establishment, the

morphological and anatomical features of the seeds responsible for these events are still unknown. For instance, the exact location of the embryo can hardly be tracked with a reasonable degree of accuracy. Thus, morphological and anatomical studies on the seed may be a necessary prerequisite to further investigate the sequence of activities involved in its germination. Knowledge about the morphological and anatomical features of the seed may help to determine appropriate methods of seed pre-treatment that may lead to quicker germination and seedling emergence.

Intentional and commercial planting of *V. paradoxa* depends on deploying an efficient system for developing its seedlings or plantlets on large-scale basis. However, success rate of the *ex vitro* vegetative propagation methods has been low (Yeboah *et al*., 2011) because the latex sap inhibits contact between the cambial cells of graft unions and also quickly blocks transpiration vessels of cut surfaces (Masters, 2002). Thus, sexual propagation still remains a reliable method of producing seedlings. Also, *in vitro* propagation techniques using juvenile plant parts with little or no latex such as the immature shoots may offer better chances of producing plantlets both in large quantities and on timely basis. Thus, the major objective of this study was to develop efficient techniques for propagating *V. paradoxa* as part of initial efforts for domesticating the plant. The specific objectives were to

i. study the morphological and anatomical features of the seed
ii. investigate the stages of seedling development
iii. develop appropriate nursery protocol for producing seedlings abundantly
iv. develop an *in vitro* protocol for regenerating *V. paradoxa* plantlets using rudimentary shoots as explants.

CHAPTER 2

2.0. Literature review

2.1. Origin and distribution

Shea tree (*Vitellaria paradoxa* C.F.Gaertn.) is of African origin growing wild in West and Central Africa (IPGRI, 2006). It is a major component of the woody flora of the Sudan and Guinea savanna vegetation zones of sub-Saharan Africa (Byakagaba *et al*., 2011). *Vitellaria paradoxa* occurs in 19 African countries namely: Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Côte d'Ivoire, Ethiopia, Ghana, Guinea, Guinea Bissau, Mali, Niger, Nigeria, Senegal, Sierra Leone, Sudan, Togo, Uganda and Democratic Republic of Congo (Hall *et al*., 1996).

Vitellaria paradoxa was first described by Mungo Park, the Scottish explorer, in 1796 in the Ségou region of Mali (Sanou and Lamien, 2011). According to Allal *et al*. (2011), West Africa has the highest genetic diversity and the densest tree populations, thereby implicating the subregion as the most probable centre of origin of the species. The Moroccan traveller Ibn Battuta had documented shea butter as a high-value commodity in regional trade across West Africa as early as 1354 (Masters, 2002). Unknown precisely is the exact location from where *Vitellaria* germplasm spread to the other African countries (IPGRI, 2006), including Ghana.

In Ghana, *V. paradoxa* grows abundantly in the northern sector and particularly thrives well in the Northern Region in Eastern Gonja, Western Dagomba, Southern Mamprusi, Western Gonja and Nanumba with Eastern Gonja having the densest stands (Lovett and Haq, 2000a) (Fig. 2.1A). In the Upper West Region, it occurs in Lawra, Tumu, Wa and Wechiau where pure stands of the trees are commonly found.

V. paradoxa grows extensively in the Guinea savanna and less abundantly in the Sudan savanna; thus, tree population in the Upper East Region is scarcely as dense as those in Northern and Upper West regions (Fobil, 2007). Sparse shea tree cover exists in Brong-Ahafo, Ashanti, Eastern and Volta regions of Southern Ghana (Fobil, 2007).

The Shea Belt, the geographical region in Africa where *Vitellaria* grows, is approximately 5000 km long and 500 km wide and ranges from western Senegal to northwestern Uganda (Shea Matters, 2011). Within the Shea Belt, the species occurs in areas with 400–1800 mm annual rainfall: its distribution area spreads from West-East Africa and up to the Adamaoua Province in Cameroon (North–South Africa) (IPGRI, 2006). *Vitellaria paradoxa* is localized between the latitudes 9° and 14° N in West Africa, 7° and 12° N in Central Africa and 2° and 8° in East Africa (Fig. 2.1B). The species is thus absent from humid forest, coastal areas and highlands at altitudes above 1600 m (Bonkoungou, 2002). It thrives well on various soils but avoids alluvial hollows and those prone to flooding (Tropical Advisory Service, 2002).

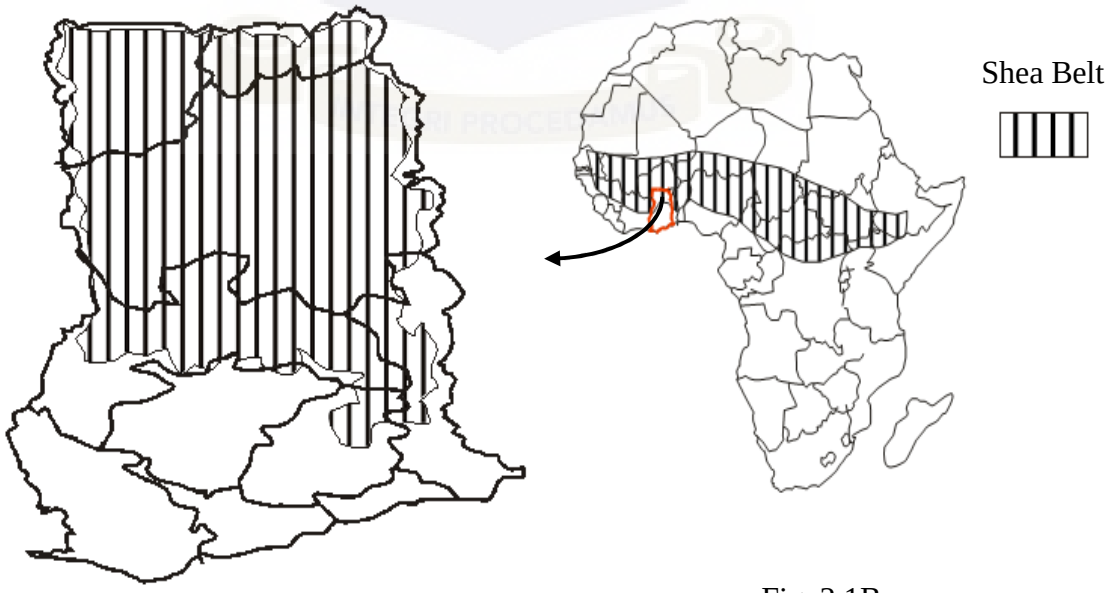

Fig. 2.1A Fig. 2.1B

Fig. 2.1. Maps showing the Shea Belt; A, Ghana's Shea Belt (Re-drawn from Quainoo *et al*., 2012); B, Native range of shea tree (Re-drawn from Hatskevich *et al*., 2011)

2.2. Taxonomy and classification

Shea tree is a bacciferous fruit tree belonging to the sapodilla family, Sapotaceae, which contain flowering plants of the order Ericales. The Sapotaceae include both evergreen and deciduous trees, shrubs and lianas in approximately 65 genera with pantropical distribution (Jessup and Short, 2011). Some sapotaceous species such as *Synsepalum dulcificum*, *Chrysophyllum albidum, C. giganteum, Tieghemella heckelii* and *Vitellaria paradoxa* all of which are indigenous to Ghana produce edible fruits, oils and timber (Hawthorne and Jongkind, 2008).

Botanically, shea tree was validly named *Vitellaria paradoxa* in 1807 by Carl von Friedrich Gaertner. Later, the butter-producing tree of West African origin was renamed *Butyrospermum parkii* (G.Don) Kotschy in 1865 in which the genus name *Butyrospermum* translates from Latin as butter (*butyros*) and *spermum* (seed) and the specific epithet *parkii* honours Mungo Park. *Butyrospermum parkii* remained as the most popular name of the species throughout the 20th century. However, *V. paradoxa* has priority and is, therefore, the botanically valid name whilst *B. parkii*, and *B. paradoxum* are homotypic synonyms (McNeill and Turland, 2011).

The genus *Vitellaria* is monotypic but has 2 subspecies known as *Vitellaria paradoxa* subsp. *paradoxa* [synonym: *Butyrospermum parkii* (G.Don) Kotschy] and subsp. *nilotica* (Kotschy) A.N.Henry, Chithra and N.C.Nair (synonym: *Butyrospermum niloticum* Kotschy) (Henry *et al*., 1983). The 2 subspecies are simply named *V. paradoxa* and *V. nilotica* respectively. Subspecies *paradoxa* has a less dense and shorter indumentum and slightly smaller flowers than those of *nilotica* (Nikiema and Umali, 2007). It occurs in West Africa whilst *nilotica* is found in East Africa with no

overlap in their ranges. However, Hall *et al*. (1996) recognize no clear-cut distinction between the subspecies' morphology and thus concluded that the difference is purely clinal. Further studies to clarify such differences would be useful for breeding.

With the wide phenotypic diversity among shea tree populations, Diarrassouba *et al*. (2009, 2008) classified the species morphologically on the basis of shape of fruit and of tree canopies, which shows some amount of discrete variation. Diarrassouba *et al*. (2009) identified 5 morphotypes based on fruit shape which are fusiform, round, ovoid, reverse pear and ellipsoid fruits, while the morphotypes reported based on canopy shape are ball or spherical, broadly pyramidal, broom and oblong-shaped trees (Fig. 2.2). Similarly, Yidana (2004) described 4 major fruit types with differences that are consistent enough to serve as basis for varietal classification and development. These phenotypic differences suggest that it is possible to select for improved performance both in fruit production and in time to fruit production.

2.3. Botany of shea tree

2.3.1. Canopy and branching

Vitellaria paradoxa is a small to medium-sized deciduous tree growing up to 7–25 m tall (Sanou and Lamien, 2011). Parkland and fallow trees are generally bigger than bushland trees (Fig. 2.2). The bole of the tree is short, usually 3–4 m long, up to 0.5–1.5 m in diameter with the bark being blackish, greyish, rough, deeply fissured and splitting regularly into corky square or rectangular scales. It copiously produces white latex which coagulates on the corky barks when cut (Hall *et al*., 1996).

Fig. 2.2. *Vitellaria paradoxa* tree in agroforestry parkland; Adapted from Diarrassouba *et al*. (2009)

Boughs and twigs of *V. paradoxa* grow plagiotropically (Diarrassouba *et al*., 2009; Hall *et al*., 1996). Due to the plagiotropic branching, *V. paradoxa* trees produce epicormic shoots (Fig. 2.3) which readily sprout from disturbed main and secondary branches. Main and secondary branches produce a large number of twigs giving rise to canopy morphotypes of varied shapes and sizes which have been described as round to spindle-, umbrella- or broom-shaped (IPGRI, 2006) (see also Section 2.2). Morphotypes based on canopy shape play an important role in tree selection and management on parklands. For example, broom-shaped trees which usually overshade annual food crops face intensive, selective thinning in agroforestry parklands (Boffa, 2000). Also, trees with round canopies have been identified as higher yielding than those with erect or oblong shapes (Schreckenberg, 1996).

Fig. 2.3. Bole of a *Vitellaria paradoxa* tree showing fissured corky bark (1), epicormic branch (2) and leaf (3); Adapted from Yidana (2004)

2.3.2. Roots

Vitellaria paradoxa has a taproot system with the taproot growing up to 1.0 to 2.0 m long. It produces shallow lateral roots that are concentrated at a depth of 0.1 m extending up to 20.0 m outwards from the tree. Secondary lateral roots are also produced, which grow downwards to the same depth as the taproot. Due to the shallow root development, mature trees are easily toppled over by strong winds occurring in the rainy season (Moore, 2008). The shallow roots also contribute to early leaf abscission; a feature that is implicated by Soro *et al*. (2012) to promote precocity (early flowering and fruiting in mature trees).

2.3.3. Leaves and flowers

The leaves of *V. paradoxa* are simple and entire with craspedodromous venation and prominent marginal veins (Fig. 2.3). They are spirally arranged in dense clusters and at the tips of the branches (Nikiema and Umali, 2007). Leaf blade measures 10–25 cm long and 4–14 cm wide with cuneate to rounded or slightly cordate base and rounded to acute apex. Leaves are both stipulate and petiolate with petioles 3–10 cm long. The greenish or cream-yellowish, fragrant flowers develop between December and March. Leaves are shed and the inflorescence appears in clusters approximately 10–40 at the shoot apex in the axils of scale leaves (Maranz and Wiesman, 2003). Flowers are complete and allogamous (Okullo *et al*., 2004). Pollination is largely entomophilous and insect pollinators include bees, wasps and ants (Cardi *et al*., 2005).

2.3.4. Fruits

The fruit of *V. paradoxa* is a berry containing 1–5 seeds (Fig. 2.4). Single-seeded fruits are the commonest and among the multiple-seeded fruits, double-seeded ones are those most frequently produced (Diarrassouba *et al*., 2009). The oval-shaped fruit is about 3–8 cm long and 2–4 cm wide and weighs between 10 and 60 g. Both fruit shape and size differ greatly among trees (Nyarko *et al.*, 2012: Yidana, 2004). The fruit is initially green and pubescent, but turns yellowish green and smells tartly strong on maturity (Nikiema and Umali, 2007). The edible pulp of the fruit comprises the epicarp and the mesocarp (Fig. 2.4C). The thicker mesocarp overlying the hilum is lined with a fibrous and bitter-tasting funiculus which is scarcely consumed. Thus, the frugivorous dispersers, mainly flying foxes (Chiroptera: Pteropodidae), pick ripe fruits from the trees, carry them in their mouths to their roosts where they eat the entire pulp (Djossa *et al*., 2008) and then disperse the seed with the funiculus intact.

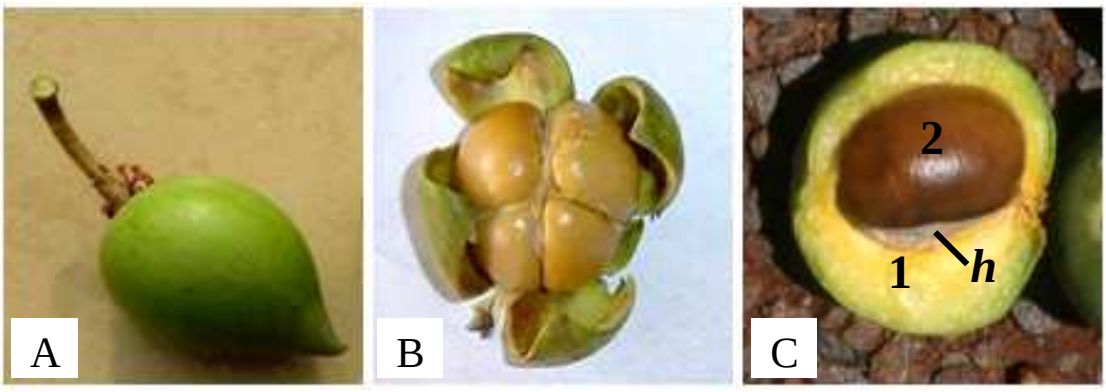

Fig. 2.4. Fruits of *Vitellaria paradoxa*; A, Fusiform-shaped shea fruit; B, Multiple-seeded shea fruit; C, Single-seeded shea fruit showing the edible pulp (1), seed (2) with its hilum (*h*); Adapted from Diarrassouba *et al*. (2009)

2.3.5. Seed anatomy and morphology

The seed of *V. paradoxa* has a fragile testa that encloses an oleaginous kernel (Orwa *et al*., 2009). The fat content of the shea seed on average is 50 % (Shea Matters, 2011). The seed or the kernel is often incorrectly called a nut (Nikiema and Umali, 2007). The incorrect naming arises from culinary sense in which any oily kernel found within a shell is termed a nut. The seed is globose or broadly ellipsoid, 3–5 cm long and 2.0–3.5 cm wide, and weighs between 5–16 g. The coat of the seed, commonly called shell, is shiny and has a conspicuous hilum, large and pale, that covers nearly one side (Jøker, 2000) (Fig. 2.4C). Colour of the shell of mature seeds is homogeneous within a given tree and may be dark brown, clear brown, greyish brown or blackish brown (Diarrassouba *et al*., 2008). The fresh kernel comprises 2 thick, fleshy cotyledons and an unexserted radicle (Nikiema and Umali, 2007).

The seeds of sapotaceous trees may be put into 2 main groups based on their morphology and the amount of latex they contain. On the basis of morphologies, the seeds are either laterally compressed or broadly ellipsoid to globoid. Examples of the laterally compressed or flattened seeds are those produced by *Chrysophyllum* spp. and

Micropholis guyanensis (Jessup and Short, 2011). The broadly ellipsoid to ovoid seeds are produced by *V. paradoxa*, C. *giganteum* and *Tieghemella heckelii*. The laterally compressed seeds, which usually contain little amount of latex, have appressed cotyledons and therefore exhibit schizocotyly. In contrast, the broadly globoid seeds which are particularly laticiferous have fused cotyledons (Watson and Dallwitz, 2012). The seeds of *V. paradoxa* have fused cotyledons and also produce latex copiously (Nikiema and Umali, 2007).

Schmidt (2000) reported that seeds are asymmetrically shaped to ensure that they scarcely fall and lie vertically (radicle end up) during dispersal because this orientation reduces the rate of seedling emergence. The morphology of sapotaceous seeds clearly suggests the orientations in which they are more likely to fall when they are naturally dispersed. The laterally compressed seeds usually fall and lie with the hilum laterally exposed whilst the ellipsoid to globoid ones usually fall and lie on the hilum. The earliest description of the germination of *Vitellaria* by Jackson (1968) mentions the hilum as the flatter side on which seeds usually fall and lie to germinate.

2.4. Germination of *Vitellaria paradoxa* seeds

Germination commences with the uptake of water by a seed and terminates when the radicle appears, or becomes visible. Subsequent events, including the mobilization of the major storage reserves, are associated with growth of the seedling. Seedlings become established when they exhaust the seed reserves. Therefore, germination, seedling growth and establishment are distinct phases with establishment marking the stage when a seedling dependent on seed reserves is transformed into a fully autotrophic plant (Bewley and Black, 1994).

Germination of *V. paradoxa* seed is described as cryptogeal (Fig. 2.5), a terminology outside those associated with the traditional scheme of germination and seedling types based on cotyledonar traits. Cryptogeal germination is the germination in which the plumule is initially pushed into the soil where it develops into a shoot which then emerges above the soil (Burrows and Stockey, 1994). Jackson (1968) observed that germination of a *V. paradoxa* seed involves the cracking of the testa at the broader end, followed by the appearance of a pseudoradicle which pushes the plumule and true radicle into the soil. In the soil, the pseudoradicle forms a bulge or swelling about 5–7 cm along its length and above the swelling, a pink-coloured shoot with scale leaves appears and grows upwards. Below the swelling is the true radicle which continuous growing downwards, becoming severalfold longer than the shoot (Ugese *et al*., 2010a). The shoot also continues its upward growth and ultimately emerges above the soil in about 2–3 months after sowing.

Fig. 2.5. Cryptogeal seedling of *Vitellaria paradoxa* showing *1*, pseudoradicle; *2*, bulge; *3*, true root; *4*, shoot; Adapted from Yidana (2004)

Vitellaria paradoxa seedlings delay to emerge above the soil even though germination of the seeds occurs within a week after sowing (Ugese *et al*., 2010a). The long period of seedling growth through the soil is what has been incorrectly described as dormancy. Consequently, much of the research work on germination of *V. paradoxa* seeds has been focused on breaking dormancy or achieving quicker emergence instead of identifying the processes involved in seedling development. The seed of *V. paradoxa* is recalcitrant (Sanou and Lamien, 2011; Pritchard *et al*., 2004) and germinates rapidly after shedding to avoid desiccation-related mortality (Orwa *et al*., 2009); thus, it has no dormancy period.

Despite being non-dormant, *V. paradoxa* seeds germinate and emerge non-uniformly. According to Ugese *et al*. (2010a), difference in seed provenances is one of the causes of variability in seedling emergence and growth. Sowing depth and seed size have also been proved to affect *Vitellaria* seed germination, emergence and growth of the resulting seedlings (Ugese *et al*., 2010b; Ugese *et al*., 2009). Although seedcoat has been implicated as the major cause of morphological dormancy in testaceous seeds (Msanga, 1998), Ugese *et al*. (2005) observed non-significant effect of the shell on the germination of *V. paradoxa* seeds. Accordingly, the causes of the long period of below-ground seedling growth still remain precisely unknown (Ugese *et al*., 2005). Nonetheless, identification of the factors responsible for this slow growth may help explain how dryland habitats are populated by recalcitrant species.

Desiccation-sensitive seeds frequently occur in aseasonal tropical forests (Tweddle *et al*., 2003). It thus remained to be explained why *V. paradoxa* that characterizes the woody flora of arid and semiarid savannah landscapes of SSA evolved desiccation-

sensitivity. This recalcitrance to storage may be explained by anatomical and morphological studies of the seed which are thus far lacking. Recalcitrant species do occur naturally in tropical drylands (Tweddle *et al*., 2003), yet little is known about their regeneration strategies (Pritchard *et al*., 2004). Some of the ecological adaptations of dryland species to desiccation-intolerance may be elucidated by detailed study of the germination of *V. paradoxa* seeds.

Farmers hardly plant *V. paradoxa*; therefore, very little information exists on its germination and on the morphology of the seedlings (Sanou and Lamien, 2011; Ugese *et al.,* 2010a). This scanty information is also highly conflicting. For example, Jøker (2000) reported that *V. paradoxa* seedlings emerge as late as 150 days after sowing whilst Yidana (2004) recorded seedling emergence in just 28 days after sowing. However, detailed information about germination and seedling morphology is crucial in determining why the shoot delays to emerge above the soil and in developing appropriate techniques for achieving quicker emergence (Ugese *et al*., 2005).

Vitellaria paradoxa seedlings are slow growing (Asante *et al*., 2012). The trade-off between survival and rapid growth usually favours survival in which the seedlings preferentially allocate more growth resources to develop long taproots and large root crowns (Jackson, 1974). According to Dillenburg *et al*. (2010), this growth pattern enables seedlings to persist in the soil seedling bank from year to year. Morris and Doak (1998) reported that species persisting in agro-ecological zones with high interannual climate variation possess high longevity. Thus, on attainment of maturity, longevity up to 200 or 300 years has been reported for *Vitellaria* trees (Jøker, 2000).

2.5. Socio-economic importance and uses of *Vitellaria paradoxa*

2.5.1. Domestic uses of *Vitellaria paradoxa*

The commercialization of shea products represents a perpetual source of income at different parts of the community chain, beginning with rural children and women who gather and process nuts to town dwellers as well as entire countries (Shea Matters, 2011). The shea fruit is consumed by people of all ages whilst the nut and butter (Fig. 2.6) are both export commodities. The pulp is also used to make beverages and jam, which are much appreciated in Mali and Burkina Faso (Sanou and Lamien, 2011).

Fig. 2.6. Sheanuts (A) and butter (B); Adapted from Moore (2008)

In the Sudano-Sahelian savanna, shea butter is the major cooking oil being the most important source of fatty acids and glycerol in the diet (Hall *et al*., 1996). The butter is an unguent with anti-microbial properties and is widely used to prepare herbal ointments. Accordingly, it is used as an anodyne to treat sprains and relieve pains, and to heal wounds quickly. As a cosmetic, it is used as a moisturizer to protect the skin against the windy and sunny weather especially during harmattan, which is usually

severe in Northern Ghana. The healing properties of shea butter are partly attributable to the presence of allantoin, a substance known to stimulate the growth of healthy tissue in ulcerous wounds (Wallace-Bruce, 1995).

Furthermore, every other part of the *Vitellaria* tree has several medicinal uses depending on the locality. For example, leaves are used to treat stomach ache in children. Extract of stem bark possesses broad spectrum antibacterial activity against clinical isolates of some gram positive pathogenic bacteria and thus demonstrates its potential to provide lead molecules for the production of novel antibiotics (Ayankunle *et al*., 2012). Roots and root bark are ground to a paste and taken orally to cure jaundice and are also used to treat diarrhoea and stomach ache.

Latex tapped from the bole is heated and mixed with palm oil to make glue which is used as a domestic adhesive (Hall *et al*., 1996). It is chewed as a gum especially by children and thus has the potential to be used industrially for making chewing gum just as chicle, latex obtained from sapotaceous species such as *Manilkara zapota*, *M. chicle*, *M. staminodella* and *M. bidentata* (Mathews, 2009).

2.5.2. International and industrial uses of sheanut and butter

Approximately 95 % of sheanuts provide an important raw material for cocoa butter equivalents (CBEs) that are used in the confectionery industries to manufacture chocolate (Masters *et al.*, 2004). Shea butter is used as a CBE because its melting point (32–45 °C) is similar to that of cocoa butter. It has high amounts of di-stearin (30 %) and some stearo-palmitine (6.5 %) which make it blend homogeneously with cocoa butter without altering its flow properties (Sanou and Lamien, 2011).

The butter also has numerous uses in the cosmetic industry. Cosmetically, the high proportion of unsaponifiable matter, consisting of 60–70 % triterpene alcohols, gives shea butter creams good penetrative, moisturizing, regenerative and anti-wrinkle properties. These properties enable the butter or creams containing it to protect the body from ultraviolet (UV) radiation. Having properties similar to those of sebum, it is used to produce lipsticks, soaps and other skincare products (Shea Matters, 2011).

The melting point of shea butter which is close to average body temperature of a healthy person (37 °C) primarily makes it a suitable base for ointments and medicines (Hall *et al*., 1996). Thus, the butter is used in pharmaceutical industries and in herbal medicines. In Ghana, it is the most widely used butter for making herbal ointments and balms. Clinical tests with patients suffering from rhinitis and having moderate to severe nasal congestion showed that shea butter may relieve nasal congestion better than conventional nasal drops (Sanou and Lamien, 2011).

2.6. Domestication of *Vitellaria paradoxa*

2.6.1. Domestication status of the species

Despite its importance and potentials, *V. paradoxa* still remains wild and is considered threatened by the World Conservation Union (Byakagaba *et al*., 2011). The major undesirable trait of the species in its wild form is probably the long gestation period, which according to Yeboah *et al*. (2011) and Yidana (2004) discourages farmers from domesticating it. Domestication of crops was not accomplished merely by gathering and even the most intensive harvesting of cereals never applied sufficient selection pressure to domesticate them fully. In contrast, deliberate planting exerts a strong selection pressure that fixes desirable alleles (Zohary, 2004). Lovett and Haq (2000b) described *Vitellaria* as a semidomesticate,

but Shea Matters (2011) considers it wild because a tradition of deliberately planting the trees scarcely exists.

In addition to the long gestation period, low and unstable prices of shea products have also been strongly opined to militate against domesticating the plant. Sheanuts have low prices which fluctuate widely both within and between seasons (Shea Matters, 2011). Thus, *Vitellaria* trees are lately being felled ruthlessly for short-term economic gains (Osei Agyeman *et al*., 2012; Masters, 2002) with a lot of nuts especially of trees far afield remaining uncollected (Shea Matters, 2011). Because farmer-domestication of plants is market-driven, remunerative and stable-pricing policy may tremendously accelerate domestication of the species.

2.6.2. Selection in agroforestry parklands

Agroforestry parkland is a mixture of naturally established trees and shrubs that farmers select for certain functions and cultivate together with staple food crops such as millet (Paul, 2012; Boffa, 2000). It is the principal agricultural system used by subsistence farmers in the Sudano-Sahelian savannas where they select for *Vitellaria* trees that compete minimally with their annual crops and for those whose fruits are both large and sweet. This anthropic selection made *Vitellaria* the dominant woody species in most West African savanna parklands (Hall *et al*., 1996) with its genetic make-up reflecting generations of unconscious selection (Lovett and Haq, 2000a).

2.6.3. Breeding for earliness

The early 1970s saw *Vitellaria* vegetable fat be announced as a CBE followed by a marked increase in interest from the pharmaceutical and cosmetics industries (Masters

et al., 2004). Consequently, a substation was established by Cocoa Research Institute of Ghana (CRIG) at Bole in 1976 to research into the botany and ecology of shea tree and to develop early bearing varieties. The institute has thus far developed improved agroforestry practices such as pruning mistletoe-infested trees, and identification and selective thinning of unproductive trees and has been disseminating them to farmers. However, no early bearing varieties have been developed (Yeboah *et al.*, 2011).

Some of the methods employed to propagate *Vitellaria* vegetatively are grafting, stem and root cutting, and layering with grafting being the most promising method (Yidana, 2004). Using stem cuttings with retained petioles and Seradix 3 powder (rooting hormone), Yeboah *et al*. (2011) readily induced faster rooting when the cuttings were set in a propagating bin. However, the major limitations of these methods are the low success (20–25 %) and reproducibility rates caused mainly by the latex sap (Masters, 2002). More importantly, the few successfully grafted and rooted cuttings grow slowly. For example, Sanou *et al*. (2004) recorded an annual growth rate of 12.6 cm for grafted plants. Thus, the seed seems to be the most reliable propagule, albeit non-uniform germination and rapid loss of viability.

2.6.4. *In vitro* propagation of *Vitellaria paradoxa*

As a result of the difficulties in conventionally propagating *V. paradoxa*, several researchers have attempted propagating the species *in vitro*. For instance, immature cotyledon explants were cultured on Murashige and Skoog (1962) basal medium amended with 2,4-Dichlorophenoxyacetic acid (2,4-D) at 0.01–1.0 mg/l in darkness. Maximum somatic embryo induction was obtained at 0.1 mg/l 2,4-D. However, the resulting plantlets were lost during weaning (CRIG, 2012).

Using leaf disc explants of *V. paradoxa*, Fotso *et al*. (2008) also successfully induced callus on a half-strength MS basal medium supplemented with 0.6 % agar, 4.5 % sucrose, 0.5 mg/l BAP and 0.5–5.0 mg/l 2,4-D. At 28 days after culture, a BAP/2,4-D combination of 0.5/3.0 mg/l respectively yielded 87.3 % callogenesis. Further, a BAP/2,4-D ratio of 0.5/2.0 mg/l produced 62.1 % embryonic calli with an average of 27 embryos per callus in 97 days after culture. Again, the resulting bipolar embryos were never successfully transformed into viable plantlets. Consequently, the exact causes of the loss of somatic embryos or the regenerated plantlets remain unknown.

Work done on miracle fruit (*S. dulcificum*), an African Sapotaceae, by Ogunsola and Ilori (2008) involved excising the micropylar end of the seeds containing the embryos. The embryos were successfully regenerated on MS medium supplemented with 0.1 mg/l Naphthaleneacetic acid (NAA) and 0.2 mg/l 6-Benylaminopurine (BAP). It may thus be possible to identify, locate and excise the embryo of *V. paradoxa* which is also a sapotaceous species for *in vitro* culture as well.

Sapotaceous species are particularly laticiferous (Watson and Dallwitz, 2012). Bhore and Preveena (2011) attributed a 100 % contamination of nodal sector and leaf disc explants in *Mimusops elengi* (an Asian Sapotaceae) to the latex produced by the species. Consequently, the high amount of latex sap and saponins in *V. paradoxa* could also be contributing factors to its recalcitrance to *in vitro* propagation. Despite these challenges, *in vitro* propagation with its potential of regenerating plantlets all-year round using explants from any part of the plant still offers prospects of producing uniform plantlets to domesticate *V. paradoxa* fully.

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CHAPTER 3

3.0. Anatomical and morphological studies on *Vitellaria paradoxa* seed

3.1. Introduction

The anatomical and morphological features of seeds influence their germination and seedling development (Flores, 2002). A typical dicotyledonous seed comprises seedcoat, embryo and food reserves stored in either the cotyledons or the endosperms. The exact locations and nature of these features vary from plant to plant, but they evolve to ensure the survival of the seed. A seed is enclosed in a fruit which protects it and also guarantees efficient dispersal of the diaspore (Leubner, 2012). The term diaspore refers to the dispersal unit, which in some plants is only the seed, whereas in others such as *V. paradoxa*, it is the fruit containing seed. The seed usually has one embryo which occasionally divides into 2 or several giving rise to zygotic polyembryony. Polyembryony may also arise from the nucellus of the embryo sac in which case it is termed adventitious polyembryony (Filovona *et al*., 2002). Both types of polyembryony produce 2 or more seedlings from 1 seed as opposed to monoembryony which results in a single seedling.

Another important feature of dicotyledonous seeds is cotyledon morphology based on which seeds are described as either schizocotylous or syncotylous. It determines the type of germination (Corby, 2008) and the optimal environmental conditions that the resulting seedlings require to develop properly (Kitajima and Fenner, 2000). Schizocotylous seeds, which have appressed cotyledons, germinate epigeally and do not tolerate shady conditions. Due to the presence of little food reserves in their cotyledons, the resulting seedlings develop photosynthetic ability rapidly, which explains their preference for well-lighted habitats. Schizocotylous seeds include the

majority of dicotyledonous seeds that store their food reserves in the endosperm. Endospermic seeds have thin and papery cotyledons (Kitajima and Fenner, 2000).

In contrast to schizocotylous seeds, syncotylous seeds have fused cotyledons and germinate crypto-hypogeally because the fusing of the cotyledons impedes their emergence from the seedcoat (Flores, 2002). These seeds usually store their food reserves in the cotyledons which are termed reserve cotyledons (Maia *et al*., 2005). The reserve cotyledons of *V. paradoxa* are fused to each other and remain so throughout germination and seedling establishment (Sanou and Lamien (2011). Also, all through these early growth and developmental stages, the thick cotyledons of *V. paradoxa* seedlings remain enclosed in the seedcoat until they are shed.

The seedcoat may be thick especially in some orthodox seeds, but it is generally thin and fragile in most recalcitrant seeds. Thicker seedcoats impose exogenous dormancy on the embryo whilst thinner ones scarcely do so (Pritchard *et al*., 2004). A thin coat (shell) surrounds a *V. paradoxa* seed similar to those reportedly found in other sapotaceous species (Roosmalen and Garcia, 2000). The Sapotaceae contain species which display both schizocotyly and syncotyly. For example, African star apple (*Chrysophyllum albidum*) has schizocotylous seeds and consequently germinates epigeally (Ehiagbonare *et al*., 2008). Seeds germinate when they absorb moisture from the surrounding medium through their coats.

The seedcoat has a micropyle through which the seed imbibes water and the radicle protrudes during germination (Gama-Arachchige *et al*., 2011). However, the radicles of some seeds protrude the seedcoat from a much larger opening called operculum

(Pérez, 2009; Flores, 2002). The micropyle or operculum may be anatropous (located on the hilar side of seed) or synatropous (located on a different side). The locations of the micropyle or operculum and the hilum on seeds strongly influence how to orient the seeds in planting holes (Schmidt, 2000; Swaminathan *et al*., 1992).

The seed of *V. paradoxa* is laticiferous and the latex and fat deposits surround the embryo making it difficult to be identified and may also explain its slow growth. This project was therefore aimed at studying the anatomical and morphological features of *Vitellaria paradoxa* seed. The specific objectives of the study were to

i. identify the location of the embryo in the seed
ii. examine the effects of the internal and external features of the seed responsible for the cryptogeal germination of the species.

3.2. Materials and methods

3.2.1. Shea fruit collection

Mature fruits were collected from agroforestry parklands at Ga and Tanina in the Wa West District of the Upper West Region of Ghana (Fig. 3.1) and transported by road to the Biotechnology Centre of the Ghana Atomic Energy Commission (GAEC). Three hundred (300) fruits were depulped manually to obtain fresh seeds which were washed using tap water. During depulping, the number of seeds per fruit was recorded. Seeds were spread under a shade for 6 hours to dry after which their anatomical and morphological features were studied using magnifying lenses and a stereomicroscope (Leica ZOOM 2000, Cole-Parmer, Wetzlar, Germany).

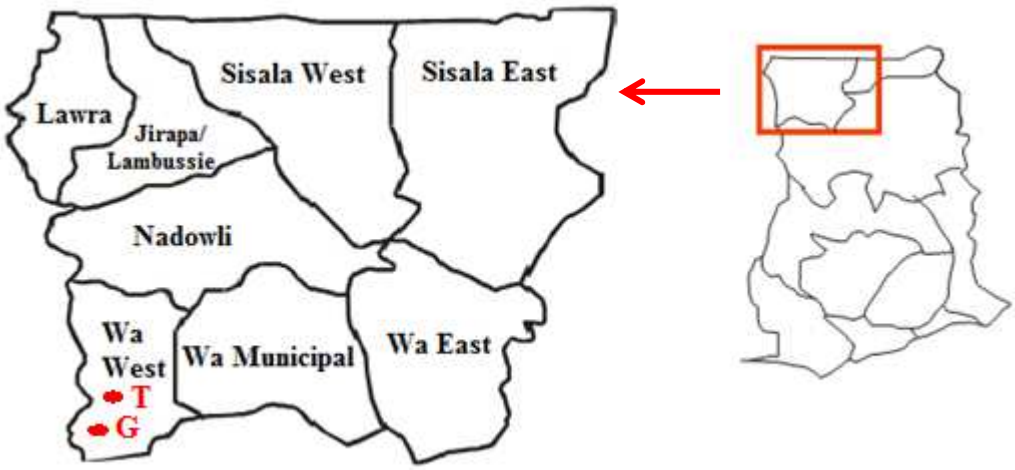

Fig. 3.1. Map of the Upper West Region of Ghana showing Tanina (T) and Ga (G) from where the shea fruits were collected

3.2.2. Studies on the morphology of *Vitellaria paradoxa* seed

The dimensions of 50 seeds were measured using vernier callipers. All dimensions were measured linearly with the length taken form the proximal to the distal end whilst the breadth and thickness were measured at the broadest part of the seed. Shape of the seed was described according to the method of Diarrassouba *et al*. (2009) whilst colour of seedcoat was described by using HTML Colour Chart (http://www.html-color-names.com/color-chart.php). Any differences in shape and other features of seeds in single- and multiple-seeded fruits were observed. The location of the micropyle in relation to the hilum was also observed.

Cotyledon morphology was studied by using 100 fresh seeds of which 50 seeds were deshelled and air-dried for 3–5 days whilst the remaining 50 were sown for 7–10 days to imbibe moisture for sprouting. Deshelling was done by using pliers to gently press the seed in the middle to rupture the seedcoat at the dorsal side. A knife was then used to remove the seedcoat (shell). Seeds were sown in polyethylene pots filled with a soil mix consisting of topsoil and well-decomposed sawdust in the ratio 5:1. The sprouted

seeds were carefully uprooted and washed using tap water, and the thickness of their pseudoradicles were measured using vernier callipers after which they were trimmed off. Both the partially dry or sprouted seeds were then split open manually by pulling apart the cotyledons beginning from the distal end. Thickness of the seedcoat was measured by cutting 40 fresh seeds longitudinally into equal parts. The kernels in the sectioned parts were gently scooped out and the thickness of the shell at the dorsal and ventral or hilar sides was measured using vernier callipers. Photographs of the observed structures were taken using a 16.1-megapexil digital still camera (Sony Corporation, China) and then translated into cartographic drawing with Microsoft Coral Drawing (Version XIII) where necessary.

3.3. Anatomical studies on the seed and embryo identification with topographical tetrazolium test

The location of the embryo was determined by using topographical tetrazolium (TTZ) test as described by Yu and Wang (1996). Six (6) fresh and 6 partially dry seeds (seeds air-dried for 72 hours) were deshelled and a quarter of their distal ends were transversely cut off. The remaining proximal portions of the seeds were washed and soaked in distilled water for 6 hours before immersion in 1.0 % tetrazolium chloride (TTC) solution in a Petri dish. The dish with its contents was wrapped tightly using parafilm and placed in a closed cabinet for 24 hours. Thereafter, the seeds were observed for staining of the embryos by the TTC solutions and photographs were taken using a Sony digital still camera (see Section 3.2.2). Twenty (20) fresh and 20 partially dry seeds were either transversely or longitudinally sectioned and the parts from where latex exuded were observed and photographed.

3.4. Results

3.4.1. Morphology of *Vitellaria paradoxa* seed

The number of seeds in a fruit varied from 1 to 5. The seed consists of 4 distinct sides termed proximal end, dorsal side, distal end and ventral or hilar side (Fig. 3.2). The distal end of the seed is comparatively smaller than the proximal end. Size of the seed is highly variable even among seeds from the same tree, with length ranging from 1.2 to 4.9 cm whilst the breadth and thickness were between 1.0–3.3 cm and 1.0–2.7 cm respectively. Seeds in single-seeded fruits were bigger than those in multiple-seeded fruits in which seed size also decreases with increasing number of seeds.

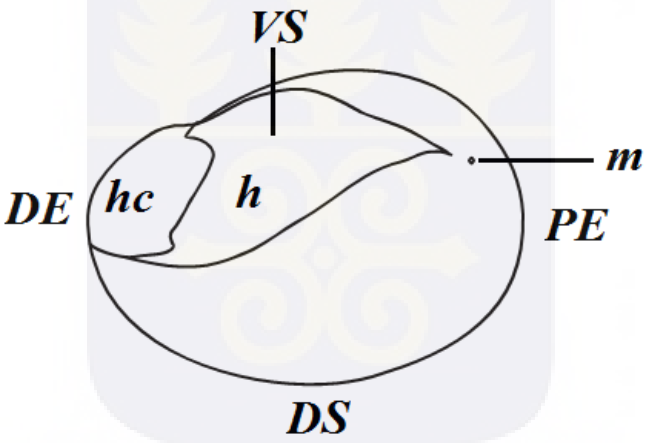

Fig. 3.2. Cartographic drawing of a *Vitellaria paradoxa* seed showing the different sides; *DE*, Distal end; *PE*, Proximal end; *DS*, Dorsal side; *VS*, Ventral or hilar side; *h*, hilum; *hc*, hilar cap; *m*, micropyle

Structurally, the seed of *V. paradoxa* comprises a coat that is flatter and slightly rough at the ventral side but smooth and convex at the dorsal side (Fig. 3.3A and B). The coat is the endocarp of the fruit wall. The ventral side comprises mainly the hilum which begins from the distal end where it is capped, broadens in the middle and tapers at the proximal end of the seed (Fig. 3.3B and D). The hilum is the flatter part of the seed with the hilar cap being completely woody (Fig. 3.3B). The shape of the hilum

varies from seed to seed, but seeds from the same parent tree have comparatively similar-shaped hila. The hila of seeds of single-seeded fruits are centrally located whilst those of multiple-seeded fruits are almost laterally located (Fig. 3.3E). The convex side of the seed is shiny whilst the hilum appears dull.

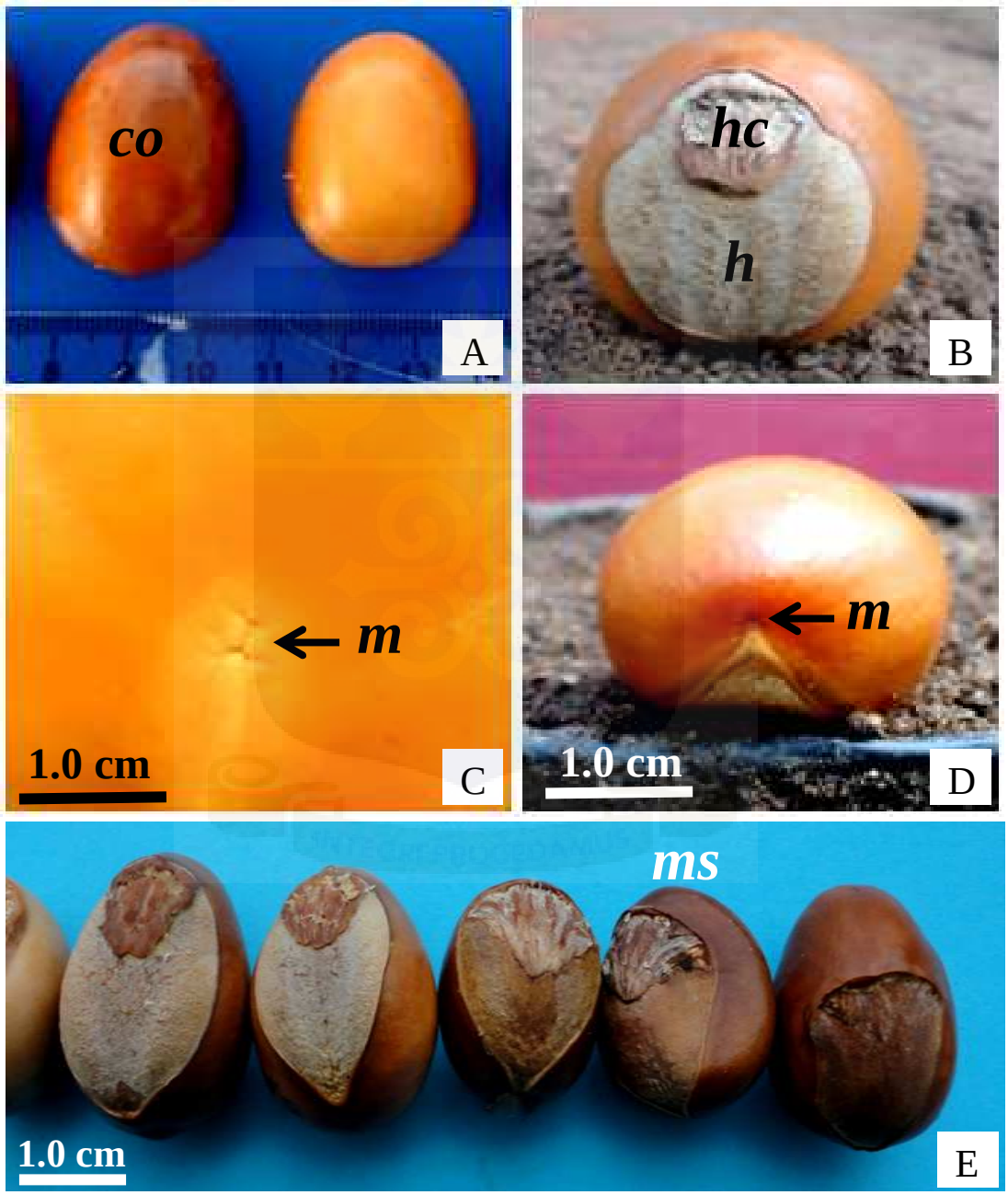

Fig. 3.3. *Vitellaria paradoxa* seeds showing A, differences in coat colour in seeds from the same parent tree; B, hilum (*h*) with the hilar cap (*hc*); C, micropyle (*m*); D, location of the micropyle (*m*); E, hila of different shapes from seeds of different parent trees; *co*, convex part of the seed; *ms*, seed from a multiple-seeded fruit

The size of the Z-shaped micropyle ranges from 0.04 to 1.00 mm (Fig. 3.3C). It is located at the proximal end of the seed where it is separated from the hilum which is at the ventral side (Fig. 3.3D). The thickness of the seedcoat varies from seed to seed, but it ranges between 0.04 and 0.07 mm at the convex part to 0.08 and 1.20 mm at the hilar side. The seedcoat is thinnest at the dorsal side where it ruptures easily with moisture loss when little pressure is exerted on it.

The colour of the seedcoat of *V. paradoxa* varies considerably even among seeds from the same tree whilst shape of the seed is comparatively the same. The colour of seedcoat is light to dark brown on the convex sides whilst that of the hilum ranges from beige to sandybrown or tan. Shape of the seed varies from spherical to globular or obovoid (Fig. 3.3A and B). Seeds in multiple-seeded fruits have flatter sides at the points of contact with each other in the fruit.

Deshelled seeds show raphes at the distal ends which are oriented either parallel or perpendicular to the embryo (Fig. 3.4A and B). Seeds whose cotyledonary raphes run parallel to the embryo were named Type 1 seeds, whilst those with their raphes perpendicular to the embryo were termed Type 2 seeds. The size of the cotyledonary raphe increases as the moisture content of the seed decreases and this desiccation creates a depression in between the cotyledons. A fully split-open seed whether air dried or sprouted shows 2 distinct sections on its cotyledons (Fig. 3.4C). One of the sections broadens at the distal part but narrows sharply and ends bluntly just close to the proximal end. This section represents where the seed is schizocotylous (the cotyledons are appressed or adpressed to each other). The other section, located at the

proximal sides, is where the seed is syncotylous (cotyledons are fused to each other). Thickness of the pseudoradicles in sprouted seeds ranged from 4 to 7 mm.

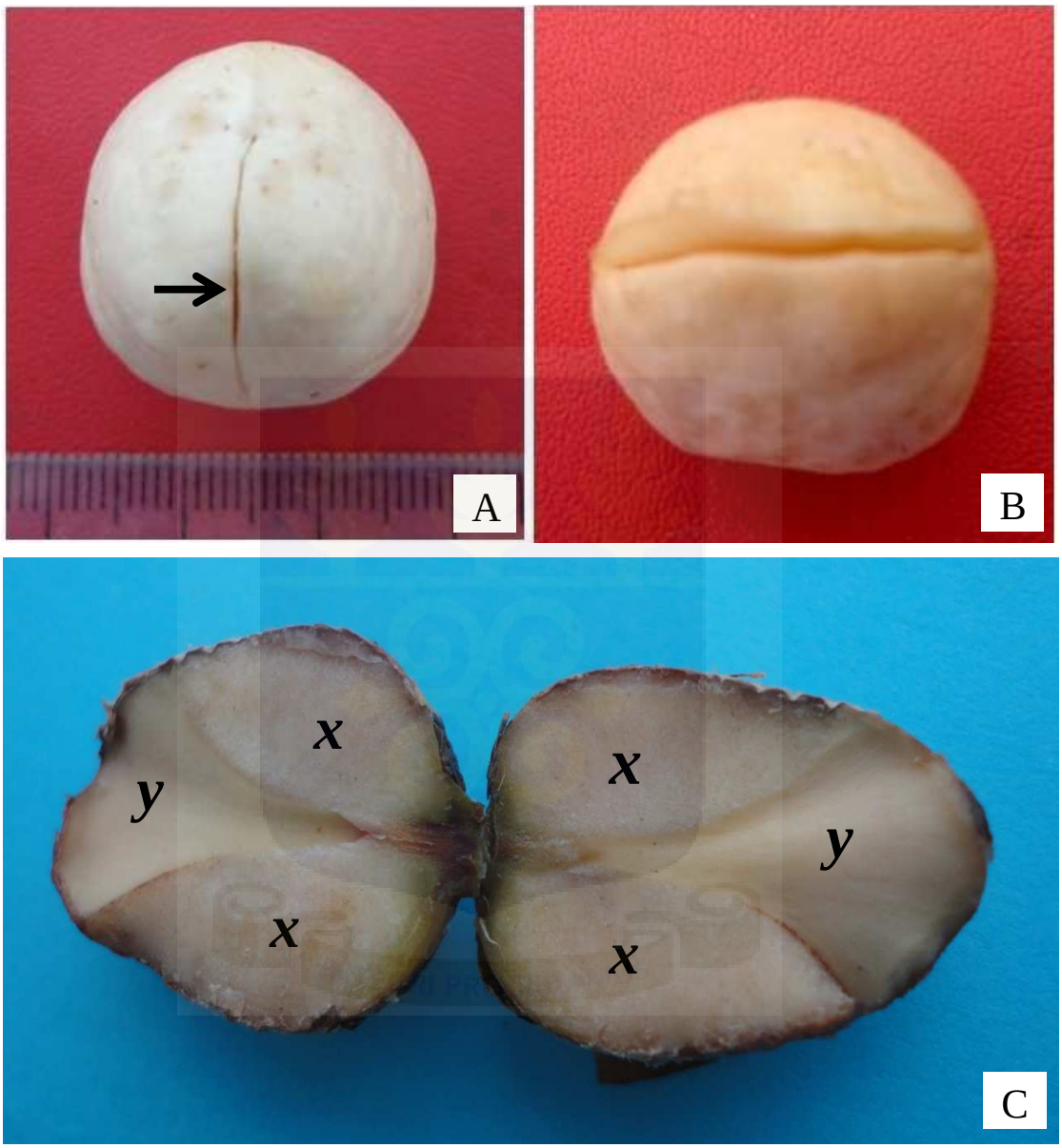

Fig. 3.4. Cotyledon morphology of *Vitellaria paradoxa* seed; A, Type 1 seed with a raphe (arrowed) parallel to the embryo; B, Type 2 seed with a raphe perpendicular to the embryo; C, Sprouted seed which is split showing where cotyledons are appressed (*y*) and fused (*x*) to each other

3.4.2. Anatomy of *Vitellaria paradoxa* seed

In a partially dry seed, the kernel shrank and pulled away from the seedcoat creating a space at the dorsal side whilst it remained appressed to the seedcoat at the hilar side (Fig. 3.5A). The cut surfaces of such partially dry kernels looked slightly pinkish or light brown depending on the seeds and their desiccation status with few drops of water-like fluids appearing on them (Fig. 3.5B). The colour of the kernels of fresh seeds is white, but it changes to light brown as the kernels lose moisture. Kernels are smooth all round with slight depressions at the raphes but appear slightly rough as they lose moisture. The raphe, visible at the distal end of the seed (Fig. 3.4A and B), widens inside the seed due to moisture loss (Fig. 3.5B).

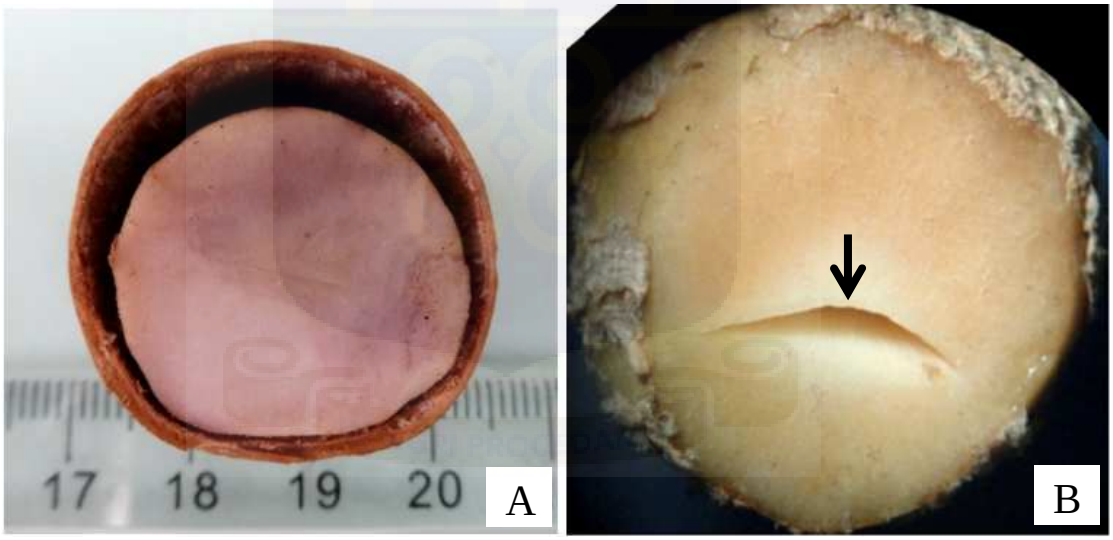

Fig. 3.5. Transverse sections through partially dry *V. paradoxa* seeds; A, Space created between the kernel and convex parts of the testa after moisture loss; B, Microscopic view (×10) of the surface of a partially dry seed showing a wider cotyledonary raphe (arrowed)

All the fresh seeds immersed in the TTC solution were stained by the tetrazolium chloride (Fig. 3.6A–C). They showed a red coloration in the slit along the 2 cotyledons with the staining being deeper towards the proximal end and visible at the

exserted spot (Fig. 3.6A and B). A longitudinal section through a stained seed showed 2 differentially stained regions (Fig. 3.6C); the lighter portion is the radicle whilst the deeper part is the plumule. Embryos, the only metabolically active parts of seeds, contain dehydrogenase enzymes which release hydrogen that reduces tetrazolium chloride (with a pale yellow colour) to a bright-red formazan (Yu and Wang, 1996). Therefore, the deeply red-stained region was considered as the embryo. Partially dry seeds did not show any red coloration indicating that they were dead (Fig. 3.6D).

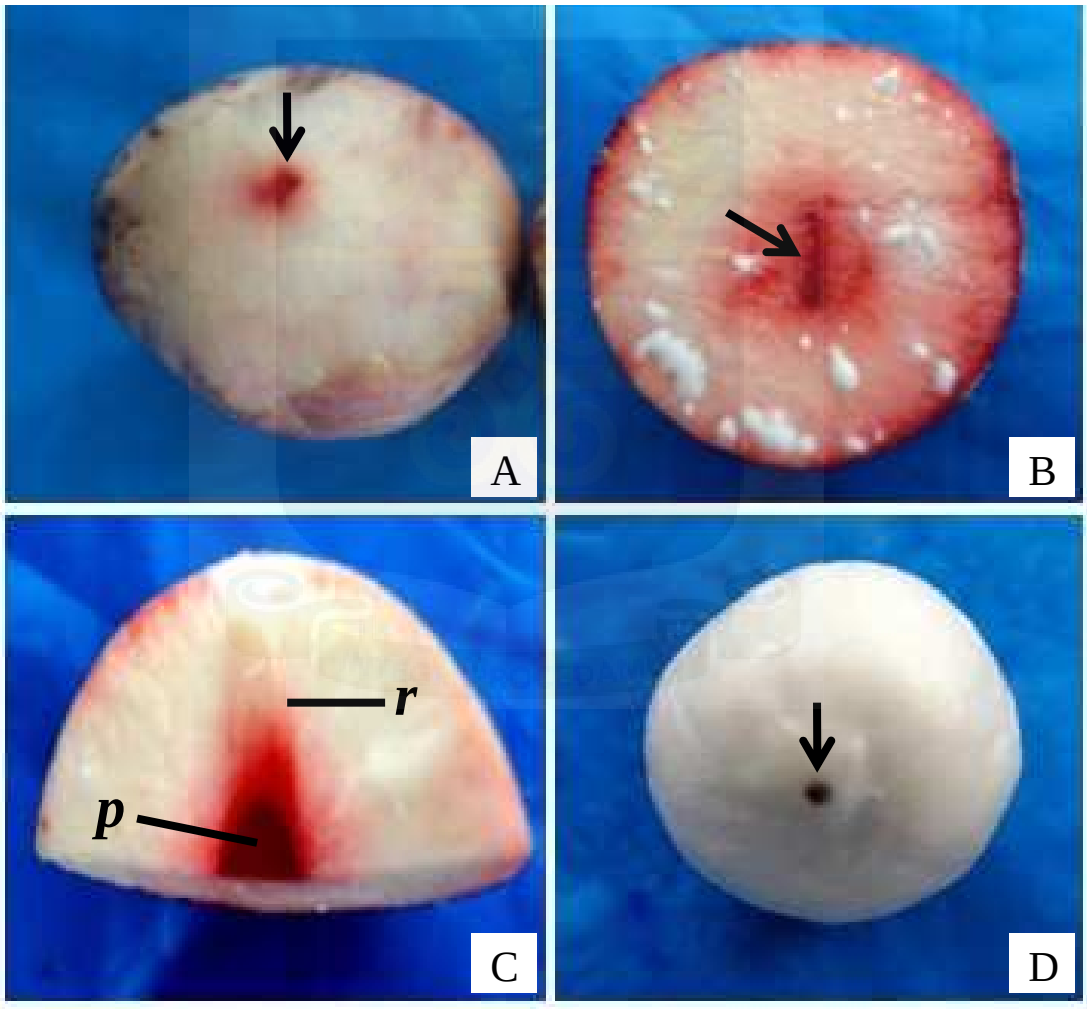

Fig. 3.6. *Vitellaria paradoxa* seeds stained by tetrazolium chloride; A, Deep red stain (arrowed) indicating the presence of a live embryo; B, Transverse section through a stained seed showing the raphe (arrowed); C, Longitudinal section through a stained seed showing two differentially stained regions: *r*, radicle; *p*, plumule; D, Dark embryo spot (arrowed) showing a dead embryo

When fresh seeds were deshelled, the spot indicated by the TTC staining as the embryo is normally light yellow (Fig. 3.7A). The light yellow spot (embryo) is either exserted or unexserted and in this study, the exserted embryos were bigger than the unexserted ones. The embryos are usually located at the proximal end, but in some seeds they were observed in other parts (Fig. 3.7B). Seeds having one embryo were considered monoembryonic. In sprouted seeds, the cotyledons usually swelled and distended at the point where the embryo is located (Fig. 3.7B).

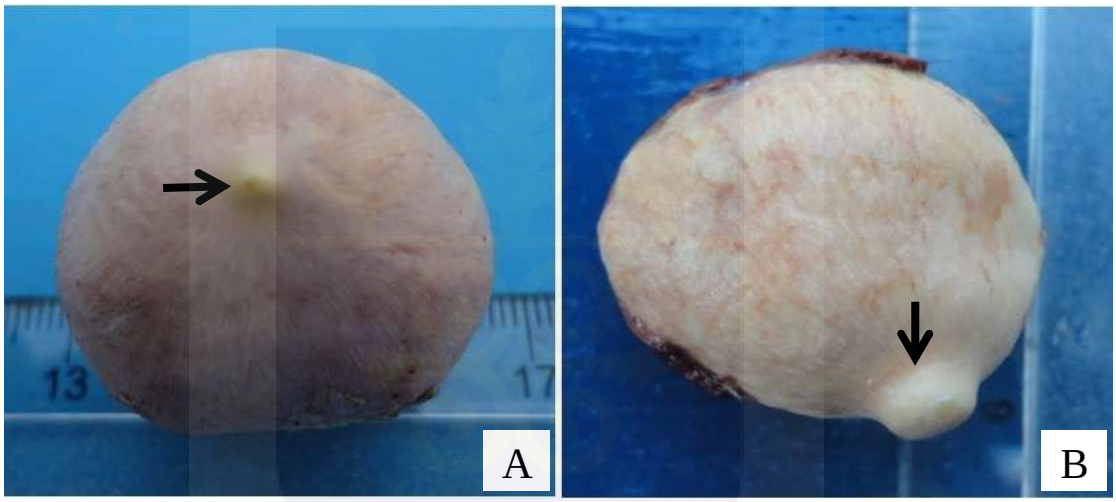

Fig. 3.7. Location of the embryo in *Vitellaria paradoxa* seeds; A, Fresh seed showing exserted embryo (arrowed) at the proximal end; B, Sprouted seed showing cotyledons distended into a 6 mm thick pseudoradicle (arrowed) at a lateral side

Some seeds had 2 yellow spots (embryos) located at their proximal ends (Fig. 3.8E) and were considered polyembryonic. The polyembryonic seeds were large with their linear dimensions ranging from 3.7–4.2 × 3.0–3.5 cm. These seeds were flat with their dorsal parts scarcely convexed. In contrast to monoembryonic seeds, polyembryonic seeds have 3 cotyledonary raphes (1 central and 2 lateral) and could be split open easily into 2 parts along the central raphe with 1 embryo located in each of them (Fig. 3.8B). Along the 2 lateral raphes, a polyembryonic seed could be split into

4 parts suggesting that each of these seeds had 4 cotyledons (Fig. 3.8B). As the moisture content of the seeds decreased, the embryos turned light brown to dark.

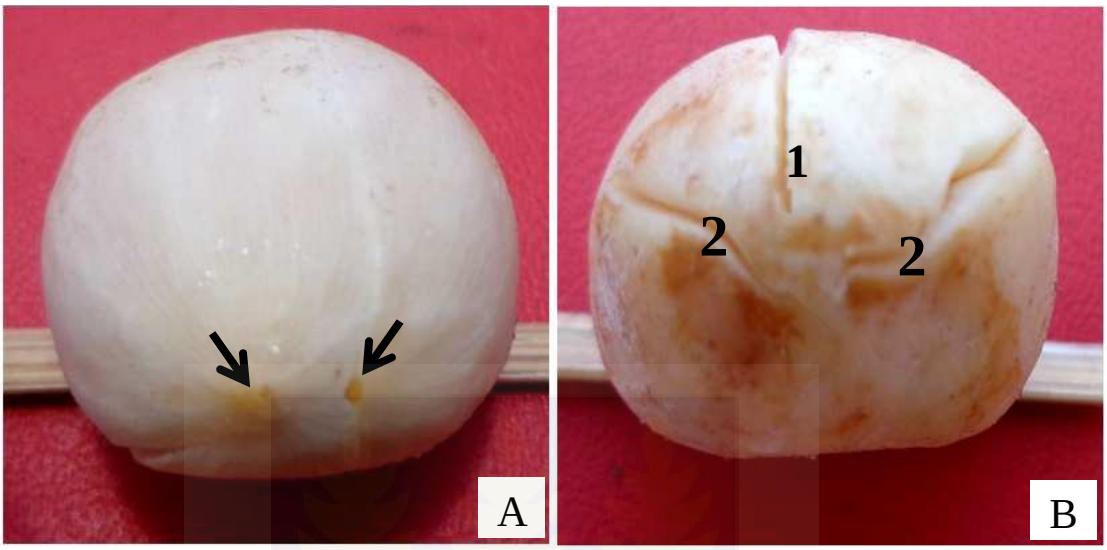

Fig. 3.8. Polyembryonic *Vitellaria paradoxa* seed; A, Proximal end of the seed showing two embryos (arrowed); B, Distal end of the seed showing the central (1) and lateral (2) raphes

Fresh cotyledons of the seeds used for this study were usually unequal with the embryo appearing as a yellow thrusted spot at the proximal end of the smaller one and its notch on the bigger one (Fig. 3.9A). In contrast to the seeds used for the TTZ test, the freshly split open seeds did not reveal embryos being well differentiated into plumules and radicles (Fig. 3.9A). For instance, when these freshly split open seeds were observed under a stereomicroscope, their plumules were never clearly distinct from their radicles. Instead, the entire embryo was seen as small and linear, and surrounded by 2 large cotyledons (Fig. 3.9B).

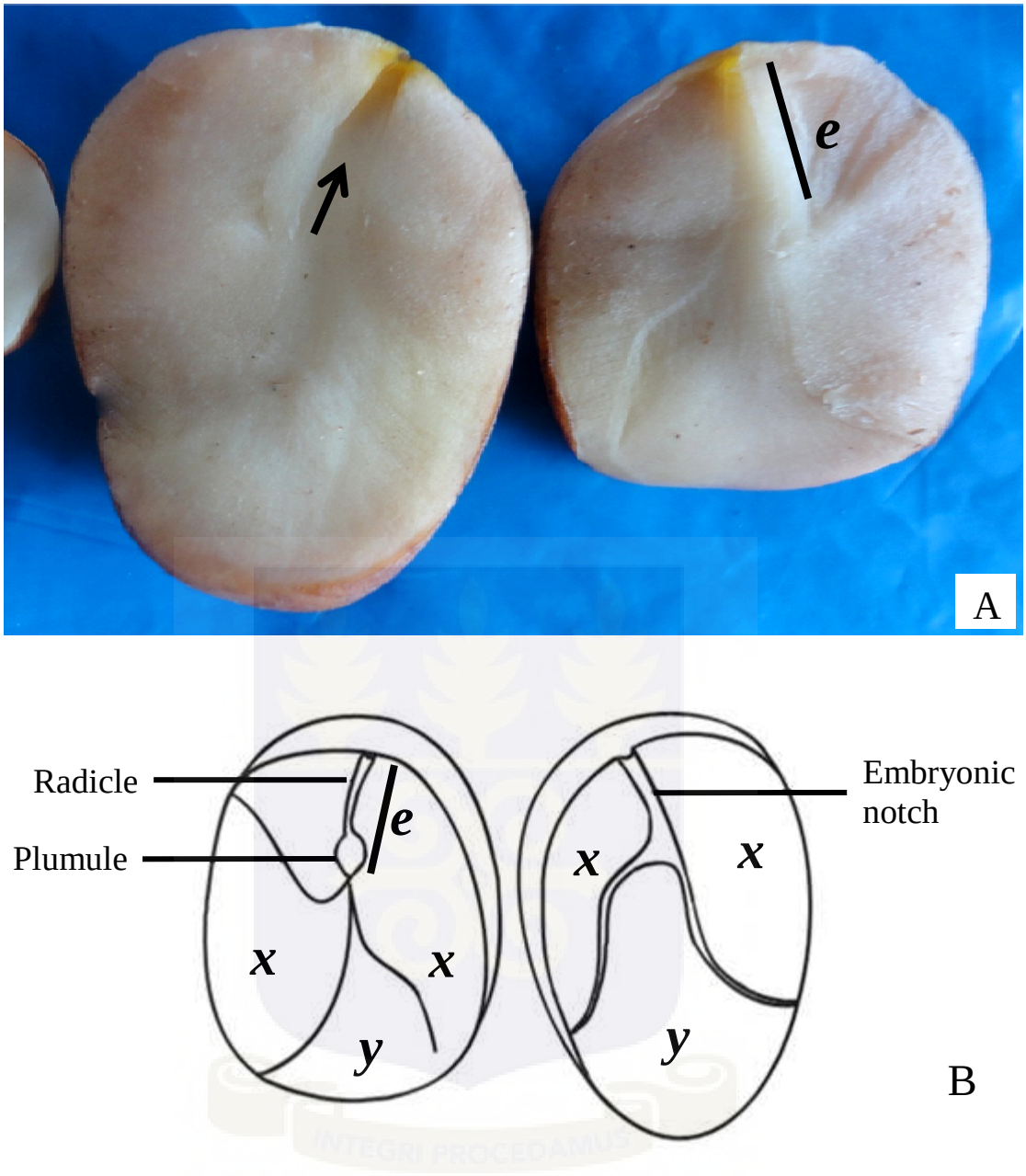

Fig. 3.9. Split cotyledons of a *Vitellaria paradoxa* seed; A, Fresh cotyledons showing the embryo notch (arrowed) and embryo (*e*); B, Cartographic drawing showing the embryo (*e*) and where cotyledons are free (y) and fused (*x*)

When fresh seeds were longitudinally sectioned, they copiously exuded latex only from the spot where the embryo is located (Fig. 3.10A). Conversely, transversely sectioned seeds exuded latex from the edges as well as from the slit in between the cotyledons (Fig. 3.10B). Partially dry seeds never yielded any latex on their cut surfaces when sectioned in either direction.

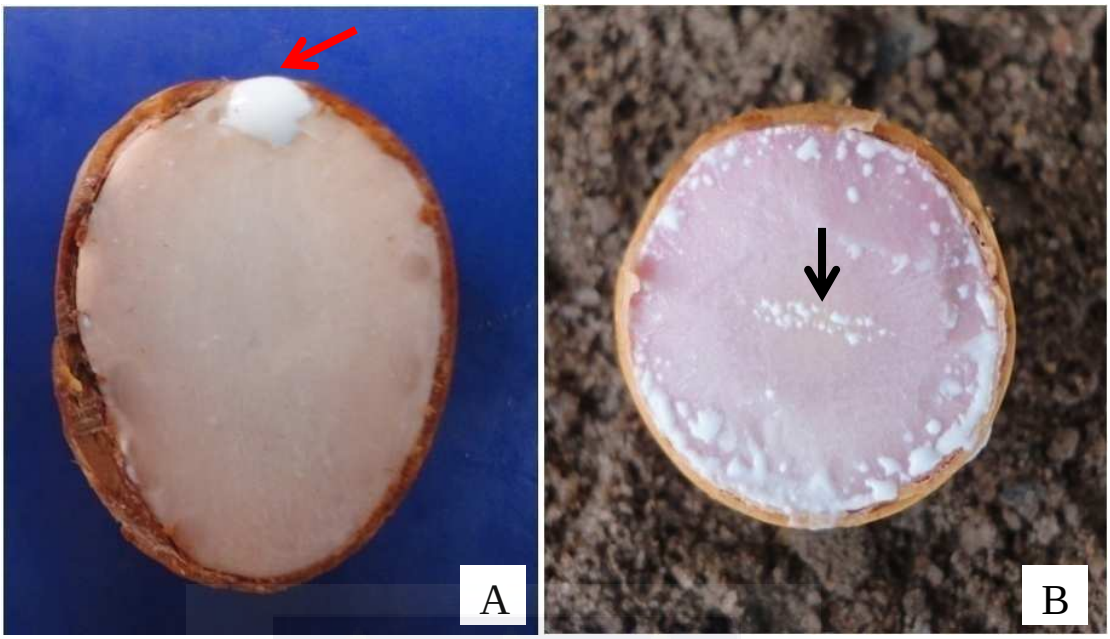

Fig. 3.10. Exudation of latex from fresh *Vitellaria paradoxa* seed; A, Longitudinal section through a seed showing latex oozing at the embryo site; B, Transverse section showing latex oozing around the edges and slit (arrowed) in between the cotyledons

3.5. Discussion

Knowledge of the anatomy and morphology of seeds is very important because these features influence germination and seedling establishment (Aguado *et al*., 2011). The convex part of a *Vitellaria* seed is smooth and shiny. The convex shape of the dorsal side confers some physiological advantage to the seed. According to Tompsett (1994), recalcitrant seeds have evolved convex shape to minimize desiccation by reducing the surface area exposed to insolation. The shiny, smooth seedcoat reflects radiant heat and thus minimizes seed desiccation. The variation in colour of coat of mature seeds from light brown to dark brown is probably due to genotypic effect of the mother tree as well as environmental conditions. In this study, variation in colour of seedcoat was observed even among seeds of the same tree contrary to the observation by Diarrassouba *et al*. (2009) who reported homogeneous coloration of seeds from the same tree at shea parkland of Tengrela Department in Côte d'Ivoire.

The micropyle of a *Vitellaria* seed is located at the proximal end, whilst the hilum is at the ventral side. This morphology most probably precludes imbibition of moisture via the micropyle. In contrast, the micropyle of many dicotyledonous seeds is located on hilar side (Schmidt, 2000). Thus, when the hilum of a *Vitellaria* seed is in contact with the soil, the micropyle is raised slightly above the soil surface (Fig. 3.3D). Because moisture uptake depends on physical contact between soil moisture and the seed, the slightly airborne micropyle can hardly imbibe moisture. Naturally, *Vitellaria* seeds are oriented hilum down during germination (Hall *et al*., 1996) suggesting that some part of the hilum exclusively absorbs moisture. The smaller size of the micropyle (0.04 to 1.00 mm) implies that the thicker pseudoradicle (4.00 to 7.00 mm) of germinating seed cannot protrude the seedcoat through it either. Thus, the Z-shaped micropyle of the *V. paradoxa* seed might be the opening on the seedcoat for only gaseous exchange.

The seedcoat is thin but the size (thickness) varied from the convex part to the hilar or ventral side. Wada *et al*. (2011) pointed out that differential thickness of the seedcoat implies differences in the sensitivities of various parts of the seedcoat to factors necessary for germination. A thin seedcoat promotes rapid germination of the seeds because it allows quick imbibition of water. According to Yidana (2004), *V. paradoxa* seeds germinate within 2–6 days after sowing. However, the thin dorsal side, which is always exposed, might also enhance moisture loss from the seed and might thus explain why extracted seed lose their germinability rapidly. Pritchard *et al*. (2004) reported that recalcitrant seeds have little physical defences such as thick seedcoat and therefore evolve rapid germination as a reproductive strategy to minimize desiccation-related mortality. Desiccation-related mortality of the *Vitellaria* seeds may also be

caused by either the kernel shrinking away from the seedcoat at the dorsal side or the widening of the cotyledonary raphes inside the seeds. Either or both of the resulting spaces may decrease the conduction of imbibed water to the embryo.

The hilum of *V. paradoxa* covers the entire ventral side running from the distal to the proximal end of the seed. The distal part of the hilum termed hilar cap is woody and appears porous and is thus the part that most likely absorbs moisture during germination. Although, many seeds imbibe water through the micropyle, a number of them absorb moisture mainly through the hilum (Xia *et al*., 2012; Maekawa, 1991) and this phenomenon may be true for *Vitellaria* seeds. All sapotaceous species thus far identified possess prominent hila (Jessup and Short, 2011; Graveson, 2009). The presence of a conspicuous hilum may be a useful taxonomic feature for identifying other members of the family particularly in tropical Africa where many plants remain undescribed and uncharacterized. According to McDonald (2013), both the texture and the area of the seed that contact the growth medium influence the rate at which water is imbibed. Thus, the flat, broad and rough hilum which is usually in contact with the soil during natural dispersal allows large soil–seed interface area which enhances rapid imbibition of water for germination and subsequent seedling growth.

The orientation of the raphes of *V. paradoxa* seeds as observed in this study revealed 2 seed types based on cotyledon morphology referred to as Type 1 and Type 2 seeds. Type 1 seeds are those in which the raphes run parallel to the embryos while Type 2 seeds, which were more common, are those in which the raphes run perpendicular to the embryos. The 2 seed types are only distinguishable when their seedcoats are removed. Cotyledon morphology is a very useful taxonomic feature in classifying

plants (Chandler, 2008) and has been used to identify different taxa in the Convulvulaceae family (Ogunwenmo, 2003). The genus *Vitellaria* is widely reported to have 2 subspecies namely *paradoxa* and *nilotica* (McNeill and Turland, 2011). However, Hall *et al*. (1996) observed no clear distinctions between the 2 subspecies. The 2 seed types with different cotyledon morphologies observed in this study may be 2 subspecies in the genus. However, this claim needs to be investigated further.

A *V. paradoxa* seed comprises 2 large cotyledons which are distally free (schizocotylous) but proximally fused (syncotylous). Whilst Nikiema and Umali (2007) reported that the seeds of *V. paradoxa* are fully syncotylous, all the seeds observed in this study were only partially syncotylous. Partial or full syncotyly influences seed germination in 2 ways. First, it impedes the emergence of cotyledons from the seedcoat during germination (Flores, 2002). Second, in syncotylous seeds, some intercalary growth at the base of the cotyledons produces petioles from which the epicotyls appear (Finneseth *et al*., 1998). Thus, the proximally syncotylous morphology of *V. paradoxa* seeds explains why they germinate crypto-hypogeally producing long and fused cotyledonary petioles which are erroneously described as pseudoradicles by Ugese *et al*. (2010) and Jackson (1974). Contrastingly, Ehiagbonare *et al*. (2008) reported epigeal germination in *Chrysophyllum albidum* (a Sapotaceae) because its seeds are fully schizocotylous.

All the fresh seeds immersed in the TTC solution stained red with the red stain more visible at the proximal ends whilst all the partially dry seeds showed no staining. Fresh or live embryos are light yellow in colour; thus, changes in the colour of the embryo to dark brown which indicated seed death was associated with a decrease in

seed moisture content confirming the desiccation sensitivity of the seeds. Embryos of seeds contain dehydrogenase enzymes whose metabolic activities release hydrogen which on contacting 2,3,5-triphenyl tetrazolium chloride reduces it to a stable, bright red triphenylformazan. According to Yu and Wang (1996), the part of the seed where red formazan is produced is the embryo. Thus, TTC staining was also used to identify the embryos for *in vitro* culture (Section 5.2.4). In some seeds, the embryos were found in different parts besides the proximal end. Therefore, in the seeds of *V. paradoxa*, the embryos could be located at different parts.

The embryo is small relative to the massive cotyledons that surround it. The TTZ test showed 2 differently stained parts identified as the radicle and plumule. Such differentiation was never observed in fresh seeds not used for the test suggesting that the seeds of *Vitellaria* most likely possess immature and rudimentary embryos. Immature embryos are commonly found in recalcitrant seeds (Berjak and Pammenter, 2008). *Vitellaria paradoxa*, therefore, might have evolved cryptogeal germination to allow the physiologically immature embryos to be pushed into the bulge of the pseudoradicle to mature before germination (producing true radicles).

Some of the *V. paradoxa* seeds used for this study had 2 embryos both located at their proximal ends (polyembryony). In contrast to monoembryonic seeds, the polyembryonic seeds could easily be separated into 2 parts along a central raphe with 1 embryo located on each of the parts. Polyembryony has been reported in many plant families and it occurs in 2 main forms namely zygotic or nucellar polyembryony (Aleza *et al*., 2010). The location of the embryos in the 2 readily separable parts of the

seeds as observed in this study suggests that the polyembryony is more likely zygotic and may result in the production of 2 seedlings per seed.

A longitudinal section through the embryo of a fresh seed resulted in copious exudation of latex only at the embryo whilst a transverse section showed latex oozing from the edges and around the cotyledonary raphe. This pattern of latex exudation suggests the presence of laticiferous vessels running parallel to one another. The laticiferous nature of the seed most likely accounts for the difficulty in isolating the embryo as well as its slow growth. In contrast, seeds of other sapotaceous species such as *Synsepalum dulcificum* scarcely show any visible latex when cut, explaining why their embryos are easily isolated for *in vitro* culture (Ogunsola and Ilori, 2008).

3.6. Conclusion

The seed of *Vitellaria paradoxa* has a shiny, brown seedcoat which is very fragile at the convex part whilst thicker at the flatter, hilar side. The shrinking of the kernel away from the seedcoat and the widening of the cotyledonary raphe reflect the recalcitrance of the seed to storage. The seed is proximally syncotylous which causes the cotyledons to swell and to elongate producing fused cotyledonary petioles during germination. It has a rudimentary embryo which is located in the small, light yellow, exserted or unexserted spot at the proximal end. The entire embryo is embedded in copious amount of latex making it difficult to be identified and isolated.

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CHAPTER 4

4.0. Germination studies on *Vitellaria paradoxa* seeds

4.1. Introduction

Germination is traditionally classified based on 3 cotyledonar traits namely position, exposition and function. On the basis of cotyledon position, germination is described as hypogeal if the cotyledons remain below the soil or epigeal if they are lifted above the soil. With reference to the exposition of cotyledons, Amritphale and Sharma (2008) classified germination as either cryptocotylar for which the cotyledons remain in the seed coat or phanerocotylar where the seed coat is shed. Using cotyledon function, Baraloto and Forget (2007) described cotyledons as reserve if they are non-photosynthetic or foliaceous if they are photosynthetic.

Considering the wide diversity of germination, Maia *et al*. (2005) described a classification system that integrates all the cotyledonar traits giving rise to 5 or 6 seedling types known as cryptocotylar hypogeal reserve (CHR), cryptocotylar epigeal reserve (CER), phanerocotylar hypogeal reserve (PHR), phanerocotylar epigeal reserve (PER), phanerocotylar epigeal foliaceous (PEF) or phanerocotylar hypogeal foliaceous (PHF). Phanerocotylar hypogeal foliaceous seedlings remain unreported or are considered as non-existent. Usually, only 1 of these seedlings types is found within a genus. Essig (1987) reported that epigeal and hypogeal germination may be used to distinguish between different subgenera within some genera.

Germination types based on cotyledonar traits lead to the understanding of seedling morphologies and culture conditions most appropriate for seedling development. Seedling types based on cotyledon morphology are used to study phylogenetic

relationships because seedling traits are highly conservative (Ibarra-Manríquez *et al*., 2001). However, no attempt has been made to describe the germination and morphology of *V. paradoxa* seedlings with reference to cotyledonar traits. Such studies may therefore be useful for both taxonomic and agronomic purposes.

Natural stands of shea trees are increasingly being cleared for farming despite the low recruitment rate of the species. Seed mortality is also rising because fruit shedding has now been coinciding with drought or dry season. These threats make it necessary to examine the germination of this economically important tree. In-depth knowledge of the germination of *V. paradoxa* will enhance nursery establishment which may eventually lead to its domestication. Thus, the major objective of this study was to examine the germination of *V. paradoxa* seeds critically as part of initial efforts towards the domestication of the plant. The specific objectives of this study were to

i. investigate the stages of development of *Vitellaria* seedlings
ii. evaluate the effect of seed size on germination and seedling establishment
iii. determine the effects of deshelling on germination and seedling growth.

4.2. Materials and methods

4.2.1. Seed collection

Shea fruits were collected as described in the previous chapter (Section 3.2.1). They were depulped manually to obtain fresh seeds which were then washed with tap water and air-dried for 6 hours. The extracted seeds were used for the various experiments unless otherwise stated.

4.2.2. Studies on *Vitellaria paradoxa* seedling development

Two hundred and forty (240) seeds of similar size were nursed in polyethylene bags filled with soil mix comprising topsoil and well-decomposed sawdust in the ratio 5:1. Seeds were treated with Hercule^{R} 50 SC (IPROCHEM Co. Ltd, Shenzhen, China) against termites and then sown 2 cm deep with the hilar side down. They and the resulting seedlings were watered as and when necessary. On sprouting, the points on the seedcoat through which the pseudoradicles protruded were observed, while the number of pseudoradicles produced per seed was counted. Five (5) seedlings were then sampled destructively at 3-day intervals beginning 5 days after sowing (DAS) to observe plumule burial in the soil, its descent to the base of the pseudoradicle and its morphogenesis into a shoot. The pseudoradicles of another set of 15 seedlings which were also sampled at the same interval and air-dried for 24, 48 or 72 hours were dissected either transversely or longitudinally to observe their anatomical features using a magnifying lens and a stereomicroscope (Leica ZOOM 2000, Cole-Parmer, Wetzlar, Germany). Both sets of seedlings were sampled 7 times each. The observed anatomical features were photographed using a 16.1-megapexil digital still camera (Sony Corporation, China). The outer covering of the pseudoradicle was manually removed to observe the core anatomical structures. The remaining seedlings which were not sampled were allowed to develop to emergence. These seedlings were observed until 3 weeks after emergence to describe the stages through which they developed. They were further classified as shrubby or multiple seedlings. A shrubby seedling developed from a single pseudoradicle but produced one or more lateral shoots, whilst a multiple seedling developed from one of the several pseudoradicles produced by a seed. Seedling morphology was described using cotyledonar traits (degree of exposition of cotyledons and their function) as proposed by Flores (2002).

4.2.3. Seed size and development of *Vitellaria paradoxa* seedlings

The linear dimensions (length, breadth and width) of 180 seeds were measured with vernier callipers. The products of these dimensions (in cm^3) were used to categorize seeds as small (13–15 cm^3), medium (19–21 cm^3) or large (25–27 cm^3). The categorized seeds were then sown on seedbeds constructed using soil mix as described earlier (Section 4.2.2). The experimental design was randomized complete block with 60 seeds in each of the 3 replicates. All seeds were sown 2 cm deep with the hilar side down as it happens in the wild. Watering and hoe-weeding were done as and when necessary. Germinating seeds and seedlings were sprayed with Cydem Super and Akape 20 SC (IPROCHEM Co. Ltd, Shenzhen, China) against insects notably termites and leaf-eating pests. Seeds were observed at a 5-day interval for signs of germination for 30 days and thereafter for emergence beginning 30 DAS. Seeds were considered as germinated when their pseudoradicles had become visible. Germination percentage and mean germination time (MGT) were calculated from the data obtained. Germination percentage was calculated as

GP = (GS*100)/ TS,

where GP = germination percentage, GS = number of germinated seeds and TS = total number of sown seeds, whilst mean germination time (MGT) was computed as

$$\text{MGT (days)} = \frac{\Sigma(t_i \times n_i)}{\Sigma n_i}$$

where t_i is the number of days beginning from the date of sowing and n_i is the number of germinated seeds at each day (Bewley and Black, 1994). Emergence percentage (EP), emergence index (EI) and emergence rate index (ERI) were computed using the formulae described by Adetimirin *et al*. (2006) as follows:

$$\text{EP} = \frac{\text{Number of emerged seedlings}}{\text{Total number of seeds sown}} \times 100\ \%$$

$$\text{EI} = \frac{\Sigma(\text{Emerged seedlings on a day})(\text{DAS})}{\text{Total number of emerged seedlings}}$$

$$\text{ERI} = \frac{\text{EI}}{\text{EP (in decimal)}}$$

Mean germination time measures the duration to sprouting, emergence index measures the rate of seedling emergence and emergence rate index estimates the duration to the emergence of all seedlings in the absence of other limiting conditions.

At bulging and emergence stages, length of pseudoradicles and taproots was measured using a metre rule. Length of pseudoradicle was measured from the seed to the cotyledonary node, whilst that of the taproot was measured from the cotyledonary node to the tip of the primary root. At emergence, root crown and shoot diameters were also measured using vernier callipers. Total shoot height (from the cotyledonary node to the tip of the shoot) was measured 3 weeks after emergence. Growth stages of *V. paradoxa* seedlings as described by Ugese *et al*. (2010) were modified and the duration of each stage was measured (in days). The 5 growth stages identified by Ugese *et al*. (2010) are sprouting, swelling of the pseudoradicle, appearance of a pink-coloured shoot on the pseudoradicle, elongation of the shoot and seedling emergence. Duration to shoot elongation was computed as the difference between the time the shoot appeared on the pseudoradicle and when the seedling emerged above the soil. Time to seedling establishment (exhaustion or transfer of seed reserve into the seedling structures) was estimated. Seed reserves were considered exhausted when the cotyledons turned dark brown both externally and internally.

4.2.4. Deshelling of seed and development of *Vitellaria paradoxa* seedlings

Freshly extracted seeds numbering 120 were used for this study. Sixty (60) seeds were deshelled whilst the remaining seeds were left intact as the control. Seeds were deshelled as described in Section 3.1.2. Both the deshelled and intact seeds were then sown 2 cm deep at a spacing of 50 × 25 cm on 3 seedbeds constructed using soil mix described in Section 4.2.2. The experimental design was randomized complete block with each of the beds as a replicate. All cultural practices performed were similar to those described in Section 4.2.3. Data were taken on germination and emergence as outlined in Section 4.2.3. Seedling growth parameters namely number of leaves, height, length and width of leaves, and stem and root crown diameters were measured at 150 and 240 DAS. Seedling height (taken from soil level) and leaf dimensions were measured with a metre rule, whilst stem diameter was measured at soil level with vernier callipers. Root crown diameters of seedlings were also measured at the widest point using vernier callipers. The linear dimensions of leaves were used to compute leaf area based on the model developed by Ugese *et al*. (2008a):

LA = 4.41 + 1.14LW,

where LA is leaf area and LW is the product of linear dimensions of the length and width at the broadest part of the leaf. Growth pattern of seedlings was described according to the terminologies of Wu and Hinckley (2010) and Tomlinson (1987).

4.2.5. Statistical analysis

Data collected were subjected to analysis of variance (ANOVA) using the Genstat statistical package (9th Edition). Percentage data on germination and on emergence were transformed using square-root transformation before analysis. Means were

separated where appropriate at 5 % significance level using least significant difference (LSD) test.

4.3. Results

4.3.1. Stages of the development of *Vitellaria paradoxa* seedlings

The development of *V. paradoxa* seedlings occurred in 7 distinct stages. These were sprouting, elongation of the pseudoradicle, bulging, shoot appearance, shoot elongation, emergence and establishment. The first 5 stages represented the skotomorphogenic growth stages because they occurred below ground whilst stages 6 and 7 were the photomorphorgenic stages because they took place above ground.

Sprouting or germination is the first stage and was marked by the protrusion of the pseudoradicle through the seedcoat. Prior to protrusion, the cotyledons swelled at the embryo side producing a thick pseudoradicle which then pushed against and ruptured the overlying seedcoat (Fig. 4.1A and B). Pseudoradicles which appeared at the proximal ends pushed against well-defined parts called opercula rupturing and sloughing the caps off (Fig. 4.1B). The operculum caps detached first at the dorsal side, but they remained attached to the seedcoat at the hilar side and were eventually pushed away by the extending pseudoradicles (Fig. 4.1C and D). The opercula and their caps were nearly circular except towards the hilar side where the boundaries were straight (Fig. 4.1C). The micropyles were still intact in the portions of the seedcoat (operculum caps) that were thrown off (Fig. 4.1D).

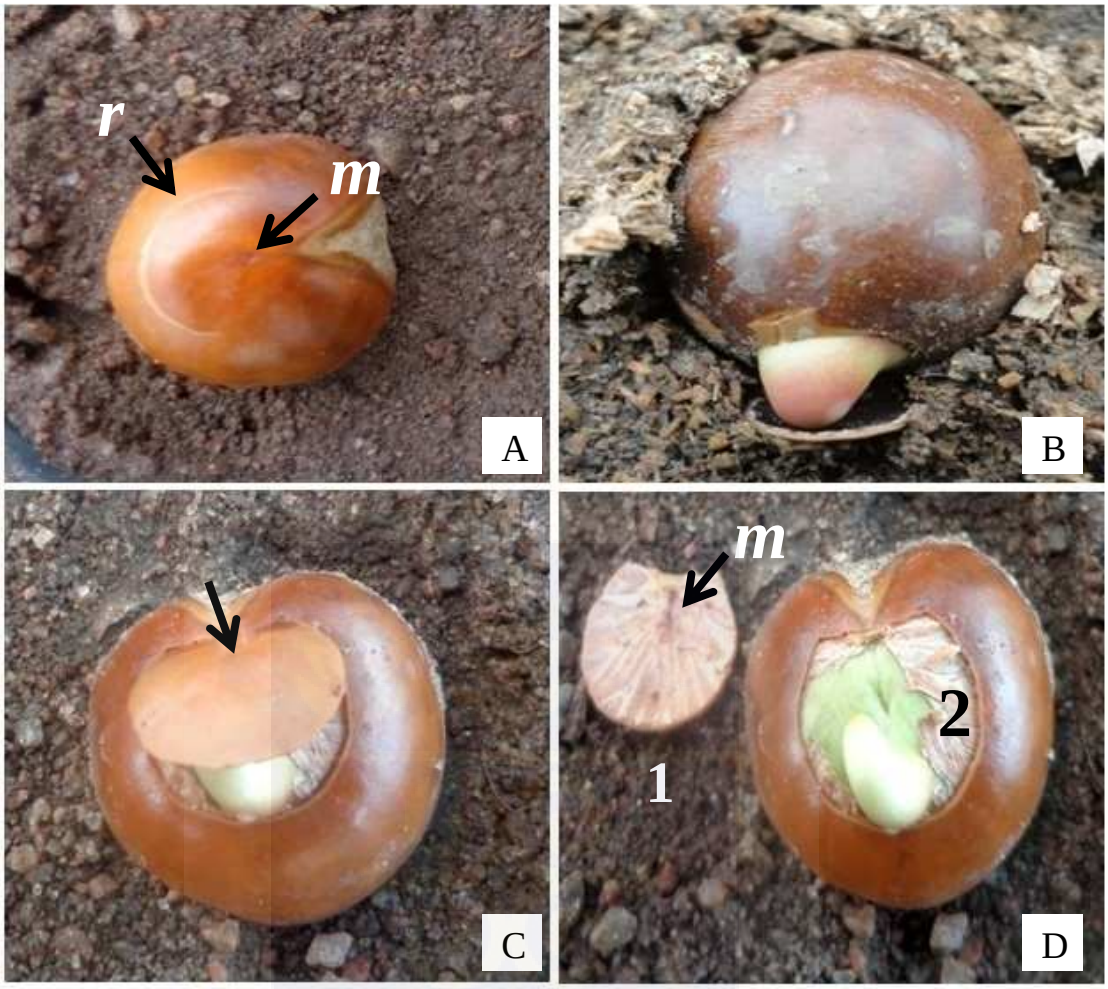

Fig. 4.1. Sprouted *Vitellaria paradoxa* seeds showing A, the margin (*r*) of the operculum and the micropyle (*m*) on the operculum; B, a pseudoradicle pushing against an operculum cap; C, ruptured operculum cap (arrowed) still attached to the seedcoat; D, micropyle (*m*), operculum cap (1) and operculum (2)

The protruding, blunt-ended pseudoradicle pushed the embryo out of the seed where it remained visible as a small light brown spot with a yellow background at the tip of the pseudoradicle (Fig. 4.2A). When the pseudoradicle became streamlined in shape, the embryo was no longer visible at the tip suggesting that it retracted into the base (Fig. 4.2B). The brittle pseudoradicle bruises easily and thereafter exudes latex profusely.

Fig. 4.2. Pseudoradicles of *Vitellaria paradoxa* seedlings at the sprouting stage; A, Embryo at the tip of a pseudoradicle; B, Streamlined shaped pseudoradicle just beginning to elongate

Ninety-two percent (92 %) of the pseudoradicles protruded through the proximal end of the seed whilst less than 4 % protruded through the other sides of the seedcoat (Table 4.1). Whereas the seedcoat ruptured along well-defined margins at the proximal end of the seed, it ruptured irregularly in distal, lateral, dorsal or ventral protrusions and often resulted in the shedding of a large part of the shell (Fig. 4.3A–E). Pseudoradicles protruding the seedcoat through the hilar side were usually thicker than those that protruded through the other sides.

Table 4.1. Protrusion of pseudoradicles from different sides of germinating *Vitellaria paradoxa* seeds

Side of pseudoradicle protrusion	Percentage
Proximal protrusion	92.0
Distal protrusion	1.2
Ventral protrusion	1.3
Dorsal protrusion	2.5
Lateral protrusion	3.0

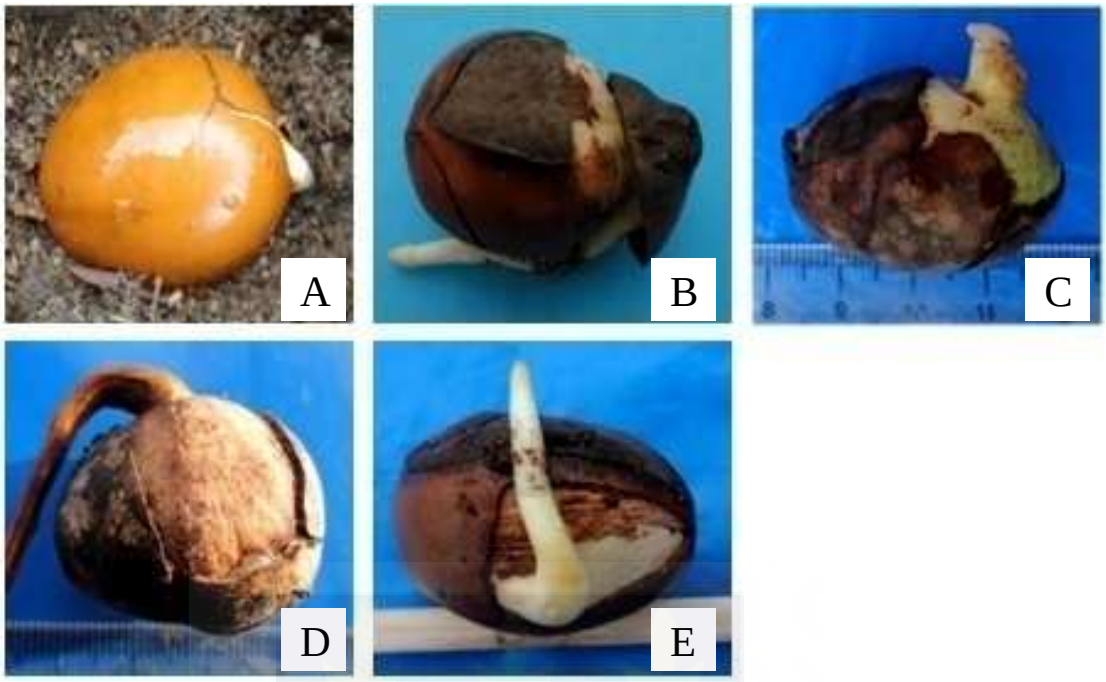

Fig. 4.3. Protrusion of pseudoradicles from different sides of germinating *Vitellaria paradoxa* seeds; A, Proximal protrusion; B, Distal protrusion; C, Ventral protrusion; D, Dorsal protrusion; E, Lateral protrusion

About 93 % of the germinated seeds produced 1 pseudoradicle each whilst the remaining seeds produced either 2 or between 6 and 10 pseudoradicles each (Table 4.2). Where 2 pseudoradicles were produced from one seed, they were similar in size but were either separated from or appressed to each other (Fig. 4.4A and B). However, closer observation of appressed pseudoradicles showed a clear line of division between them. All 6 to 10 pseudoradicles produced from individual germinated seeds were unequal in size and separated from one another (Fig. 4.4C).

Table 4.2. Number of pseudoradicles produced per germinating *Vitellaria paradoxa* seed

Number of pseudoradicles produced per seed	Percentage
1	92.5
2	0.03
> 6	7.47

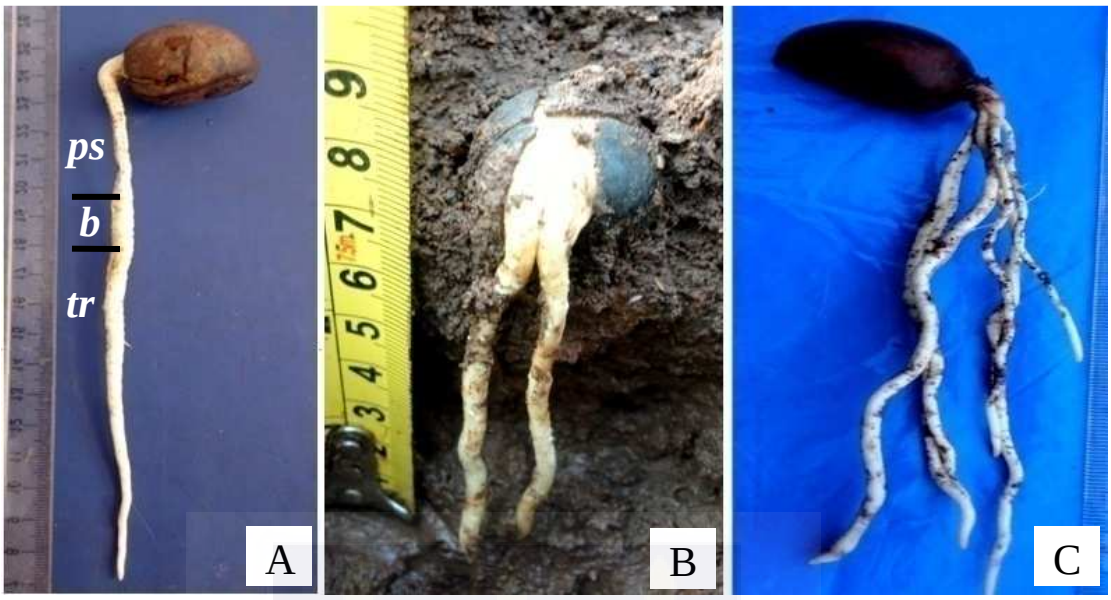

Fig. 4.4. *Vitellaria paradoxa* seedlings at the second and third developmental stages; A, Sprouted *Vitellaria paradoxa* seed showing a pseudoradicle (*ps*), bulge (*b*) and true root (*tr*); B, Two pseudoradicles produced from one seed; C, Germinated seed with 6 pseudoradicles

At the second stage of seedling development, the blunt-ended pseudoradicle became terete with the radicle visible at the tip. The pseudoradicle then elongated rapidly deep into the soil and formed a bulge or swelling at 2–8 cm along its length (Fig. 4.4A). Below the bulge, the true radicle continued its positive geotropic growth (Fig. 4.4A).

Morphologically, the pseudoradicle is smooth and cylindrical and can be split into 2 equal parts along a defined raphe with each of the parts being attached to 1 of the cotyledons (Fig. 4.5A). Removing the outer sheath of the pseudoradicle reveals laticiferous or latex-containing vessels (Fig. 4.5B). In seeds that produced either 1 or 2 pseudoradicles, each of the pseudoradicles had a central hollow tube in addition to the outer sheath and laticiferous vessels (Fig. 4.5B and C). On the contrary, all the 6

to 10 pseudoradicles produced from individual seeds had only an outer covering and a laticiferous vessel each making them solid.

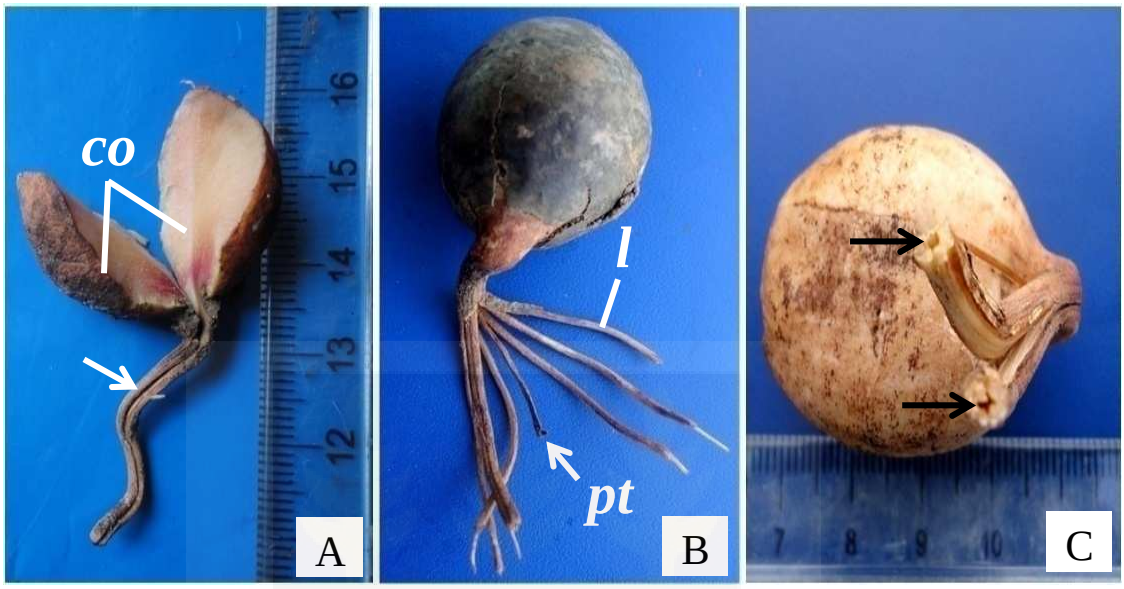

Fig. 4.5. Morphological features of the pseudoradicle; A, Cotyledons (*co*) showing the raphe (arrowed) along which their petioles were fused into a pseudoradicle; B, Pseudoradicle showing a laticiferous vessel (*l*) and the central hollow tube (*pt*); C, Seed showing two central hollow tubes (arrowed)

Transverse and longitudinal sections through the pseudoradicles showed the 2 or 3 main parts described earlier. The outer sheath enveloped the vessels into a tubular geotropic structure (Fig. 4.6A). In seeds that produced 1 or 2 pseudoradicles, the laticiferous vessels varied from 6 to 8 (Fig. 4.6A). Conversely, in seeds that produced 6 or more pseudoradicles, each of the pseudoradicles had only 1 laticiferous vessel (Fig. 4.6B). The latex vessels which extend from the seed to the base of the bulge surround the central hollow tube (Fig. 4.6A). The plumule of the embryo moves through the central hollow tube during germination until it reaches the bulge suggesting that the hollow tube may be a plumule tube (Fig. 4.7A).

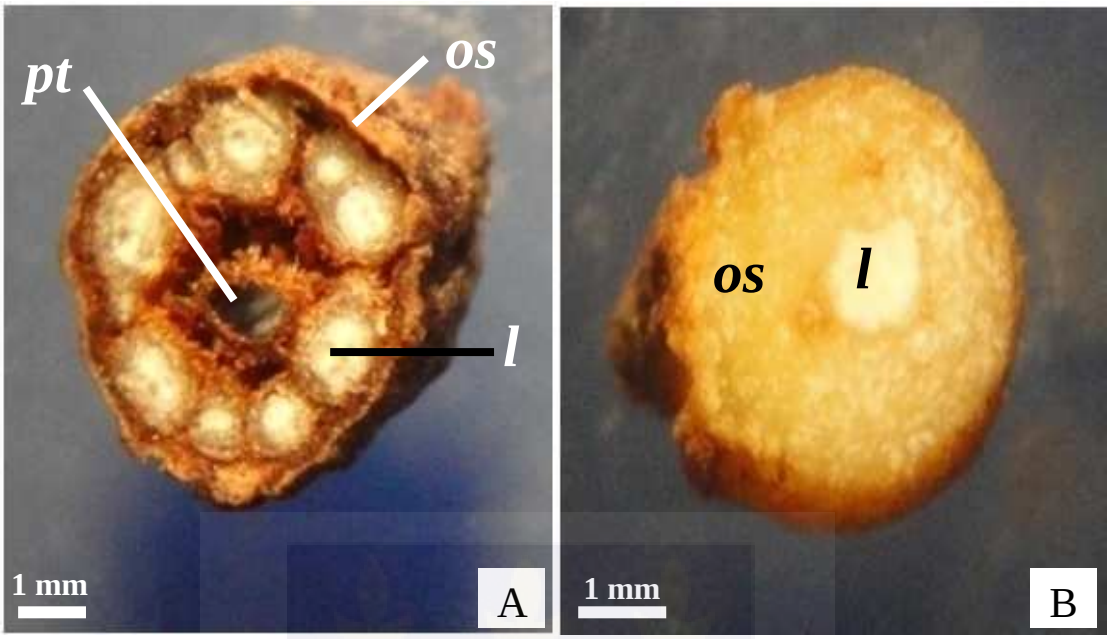

Fig. 4.6. Anatomical and morphological features of the pseudoradicle at bulging stage; A, Transverse sections showing the outer sheath (*os*), plumule tube (*pt*) and laticiferous vessel (*l*); B, Outer sheath and laticiferous vessel

The plumule which may be single or branched is surrounded by rhizoid- or hair-like structures. It is usually white but turns pink as it develops into a rudimentary shoot (Fig. 4.7B and C). Occasionally, some of the plumules never turn pink but remain white throughout the period when they develop into shoots. In this study, plumules that turned pink were more common than those than remained white. Plumules that branched developed 2 or more shoots in the bulge. The bulge, formed at the base of the pseudoradicle, is a swelling of 0.5–0.7 cm long that is produced when the descended plumule develops into the rudimentary shoot (Fig. 4.7A and C). The rudimentary shoot has internodes and nodes with scale leaves numbering 5 to 7 (Fig. 4.7D). The formation of the bulge marks the third stage of seedling development.

Fig. 4.7. Development of the rudimentary shoot at the bulging stage; A, Longitudinal section through the pseudoradicle showing the plumule tube (*pt*), descended plumule (*p*) and bulge (*b*); B, Branched plumule; C, Plumule (*p*) developing into a rudimentary shoot; D, Rudimentary shoot showing an internode (1); epicotyl (2), node (3) and scale leaf (4); Bar: D = 1.0 mm

At the fourth stage of seedling development, the rudimentary shoot then protrudes from the pseudoradicle via a cotyledonary slit (Fig 4.8A). The shoots appeared ventral, lateral (Fig. 4.8B) or dorsal (Fig. 4.8D). Occasionally, 2 or more shoots

appeared via the cotyledonary slit (Fig. 4.8C). The fifth stage is elongation of the shoot towards the soil surface (Fig. 4.8D). The shoots either elongated freely or were trapped within the pseudoradicles (Fig. 4.8B) or in-between the pseudoradicles and the seed (Fig. 4.8E). Trapped shoots could easily be freed by gently turning the pseudoradicle with the seed into another direction (Fig. 4.8F).

Fig. 4.8. *Vitellaria paradoxa* seedlings at the fourth and fifth stages of development; A, Pseudoradicle showing the cotyledonary slit (*cs*); B, Shoots that appeared lateral (1) and ventral (2); C, Three shoots that appeared ventral; D, Shoot elongating freely; E, Shoot that is trapped (arrowed); F, Trapped shoot freed by turning away the seed

The elongated shoots then emerged above the soil level marking the sixth stage of seedling development. Emerged seedlings appeared pink or light green with the pinked-coloured seedlings outnumbering those that were light green. When the cotyledons of the seedlings turned dark brown, the seedlings were considered established. At this stage, both the leaves and stems of the pink-coloured seedlings

had turned green except the growing points that still remained pink or light brown. Conversely, the light-green seedlings became fully green with their growing points still remaining light green. At the establishment stage, therefore, the seedlings had developed fully functional leaves for photosynthesis and were completely autotrophic (Appendix 7.1). Establishment is the final stage of seedling development.

Any germinated seed that produced 1 pseudoradicle also produced 1 seedling usually with a single shoot emerging above the soil (Fig. 4.9A). Occasionally, some of those germinated seeds with 1 pseudoradicle produced 2 or more shoots (Figs. 4.9B and 10B). A seedling that produced 2 or more shoots was classified as a shrubby seedling. Germinated seeds that produced 2 pseudoradicles each also produced 2 clearly identifiable and easily separable seedlings (Fig. 4.9C). Two (2) seedlings produced from 1 seed were classified as multiple seedlings. Contrastingly, sprouted seeds that produced 6 or more pseudoradicles never produced any seedlings at all.

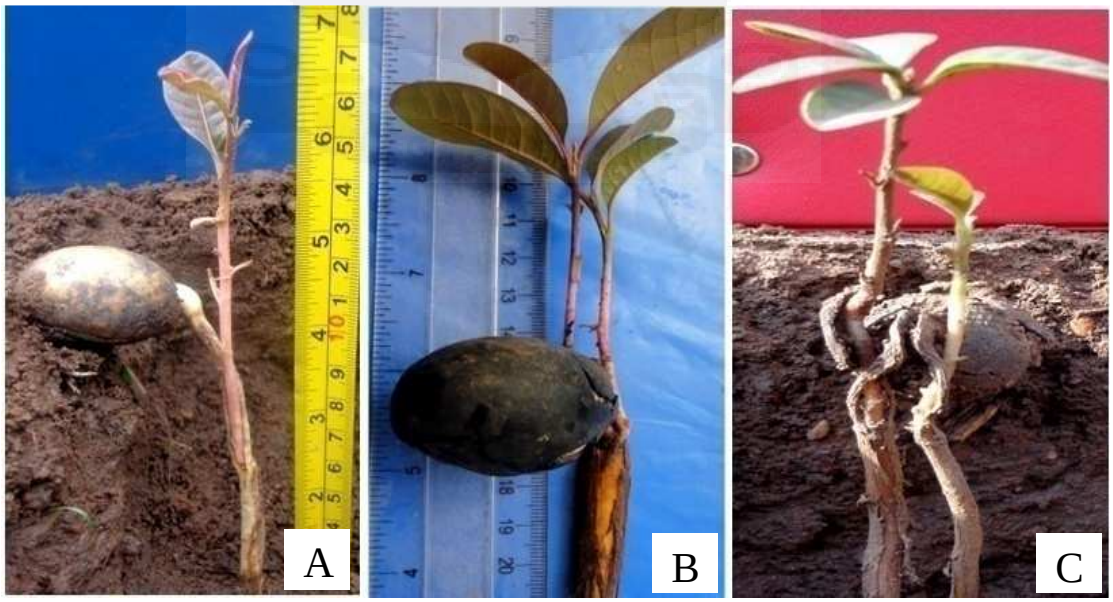

Fig. 4.9. Production of multiple shoots and seedlings in *Vitellaria paradoxa*; A, Seedling with one main axis; B, Seedling with two shoots; C, Two seedlings produced from one seed

Seedlings were further classified as either cryptocotylar or phanerocotylar depending on the degree of cotyledon exposure. In those seeds whose cotyledons were lateral to each other when sown in the hilum down orientation, the resulting seedlings were the phanerocotylar type (Fig. 4.10A). In these seeds, the cotyledons usually split-open distally, but remained slightly proximally fused. For those seeds whose cotyledons lie on top of each other in the hilum down orientation, seedling morphology was cryptocotylar or semicryptocotylar (Fig. 4.10B and C). However, all the seedlings had reserve cotyledons irrespective of cotyledon arrangement.

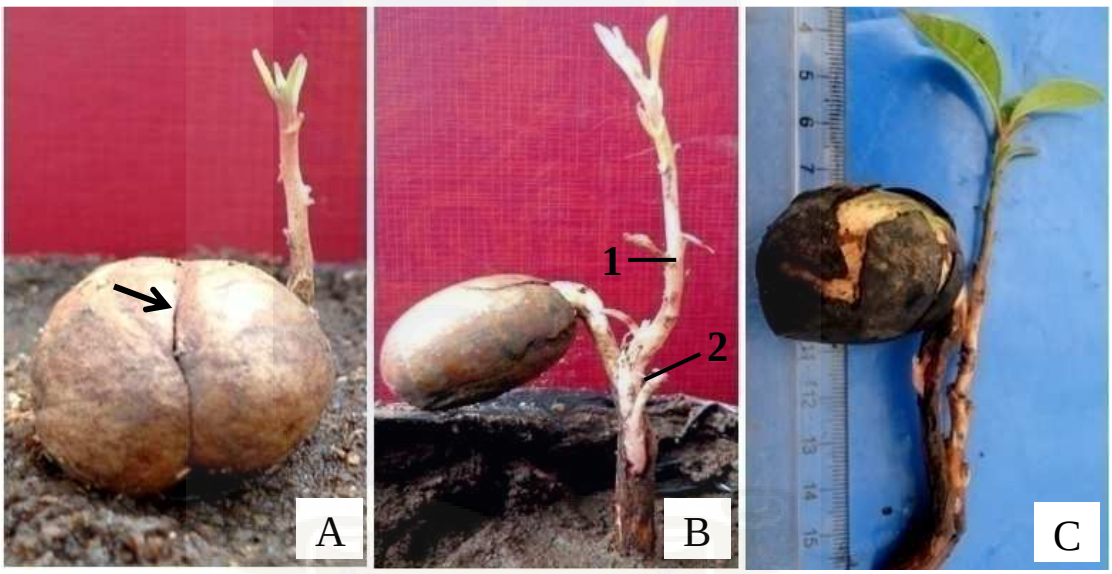

Fig. 4.10. Types of seedlings produced by *Vitellaria paradoxa* based on cotyledon exposition; A, Phanerocotylar seedling showing cotyledons with their raphe (arrowed); B, Cryptocotylar seedling with two shoots (1 and 2); C, Semicryptocotylar seedling

4.3.2. Effect of seed size on germination and emergence of *V. paradoxa* seedlings

Seed size had a significant influence on the duration to germination, pseudoradicle elongation, bulging, shoot appearance, shoot elongation, emergence and establishment of *Vitellaria* seedlings (Tables 4.3 and 4.4). All the small and medium seeds sprouted or germinated whilst 95 % of the large seeds sprouted indicating that seed size did not

have significant ($P > 0.05$) effect on germination. Similarly, emergence percentage, varying non-significantly from 93.15 % for medium seeds to 95.79 % for large seeds, was high among all the seedlings produced (Table 4.3).

Duration to sprouting varied significantly ($P < 0.05$) from 7 days in large seeds to 12 days in the small seeds (Table 4.4.). Just as sprouting, the rate of pseudoradicle elongation was also statistically different among the seeds. The pseudoradicles elongated fully among the large seeds in 12 days which was significantly ($P < 0.05$) faster than those of both medium seeds (16 days) and small seeds (22 days).

Days to shoot elongation (SE) which is the difference between days to emergence of seedlings above the soil and days to shoot appearance on the pseudoradicle (SA) had the longest duration of all the skotomorphogenic, or below-ground growth stages of *Vitellaria* seedlings (Table 4.4). It took 38 days in the seedlings produced from small seeds but followed no clearly defined trend because the shoots of the seedlings of medium seeds elongated earlier than those of large seeds.

Seedlings produced by large seeds had the shortest duration to emergence (61 days), followed by seedlings produced by medium seeds (65 days) and finally those produced by small seeds (75 days) (Table 4.4). Seed size, therefore, had a significant effect ($P < 0.05$) on days to emergence of *V. paradoxa* seedlings. The corresponding emergence rate indices of seedlings produced by large and medium seeds (65 and 70 days respectively) were statistically the same, but both were significantly shorter than that of seedlings of small seeds (78 days) (Table 4.3). Time to seedling establishment

varied significantly from 97 days for seedlings of small seeds to 99 and 114 days for seedlings of medium and large seeds respectively (Table 4.4).

Table 4.3. Effect of seed size on germination, emergence percentage and emergence rate index of *Vitellaria paradoxa* seedlings

Size class	Germination percentage	Emergence percentage	Emergence rate index
Small	100a	95.79a	78.15b
Medium	100a	93.15a	70.25a
Large	95a	93.30a	65.07a

Means in the same column followed by the same letters are not significantly different ($P < 0.05$)

Table 4.4. Effect of seed size on development of *Vitellaria paradoxa* seedlings

Seed size	Days to						
	Sprouting	PRE	Bulging	SA	*SE	Emergence	EST
Small	11.52c	21.52c	27.86c	37.99c	37.03	75.02b	97.42a
Medium	9.15b	15.89b	25.69b	33.31b	32.16	65.47a	99.29a
Large	6.98a	11.55a	20.76a	25.36a	35.34	60.70a	114.26b
Mean	9.22	16.32	24.75	32.2	34.84	67.06	103.66

Means in the same column followed by the same letters are not significantly different ($P < 0.05$); PRE, Pseudoradicle elongation; SA, shoot appearance; SE, Shoot elongation; EST, Establishment and *SE = Emergence – SA

The morphological features of *V. paradoxa* seedlings (Fig. 4.11) varied significantly as seed sized increased. The mean length of the pseudoradicles produced by small seeds was longer (6.75 cm) than those produced by medium seeds (5.12 cm) and large seeds (3.64 cm). These differences indicated that seed size had a significant ($P < 0.05$) influence on the length of the pseudoradicle (Table 4.5). Similarly, at the bulging stage, the difference between the mean length of taproot produced by seedlings of small seeds (10.29 cm) and that of those produced by medium seeds (7.65 cm) was

significant. Although length of the taproot decreased as seed size increased, no significant difference existed between the length of taproot of seedlings produced by medium seeds (7.65 cm) and that of the seedlings produced by large seeds (6.73 cm). At the emergence stage, length of taproot also decreased as seed size increased with seedlings of small seeds producing significantly the longest taproots (31.61 cm).

Table 4.5. Effects of seed size on morphological features of *Vitellaria paradoxa* seedlings at bulging and at emergence

Size class	Length of			Shoot height	Diameter of	
	pseudoradicle	taproot at bulging	taproot at emergence		root collar	shoot
Small	6.75c	10.29b	31.61c	8.45b	0.42c	0.15b
Medium	5.12b	7.65a	24.15b	9.20ab	0.56b	0.19b
Large	3.64a	6.37a	18.05a	10.05a	0.68a	0.25a
Mean	5.17	8.10	24.60	9.23	0.55	0.20

Means in the same column followed by the same letters are not significantly different ($P < 0.05$)

Contrary to length of pseudoradicle and taproot at bulging and at emergence, total shoot height increased significantly as seed size increased. The mean total shoot height of seedlings (Fig. 4.10C) produced by large seeds (10.05 cm) was significantly higher than that produced by seedlings of small seeds (8.45 cm) (Table 4.5). Similarly, at emergence stage, seed size had a significant ($P < 0.05$) effect on mean diameters of root collar and shoot because both growth parameters increased as seed size increased. Root collar diameter of seedlings of large seeds (0.68 cm) was wider than those of seedlings of medium seeds (0.56 cm) and small seeds (0.42 cm). Shoot diameter of seedlings produced by large seeds (0.25 cm) was significantly wider than that of seedlings of medium seeds (0.19 cm). However, no significant difference existed between shoot diameters of seedlings of medium and small seeds.

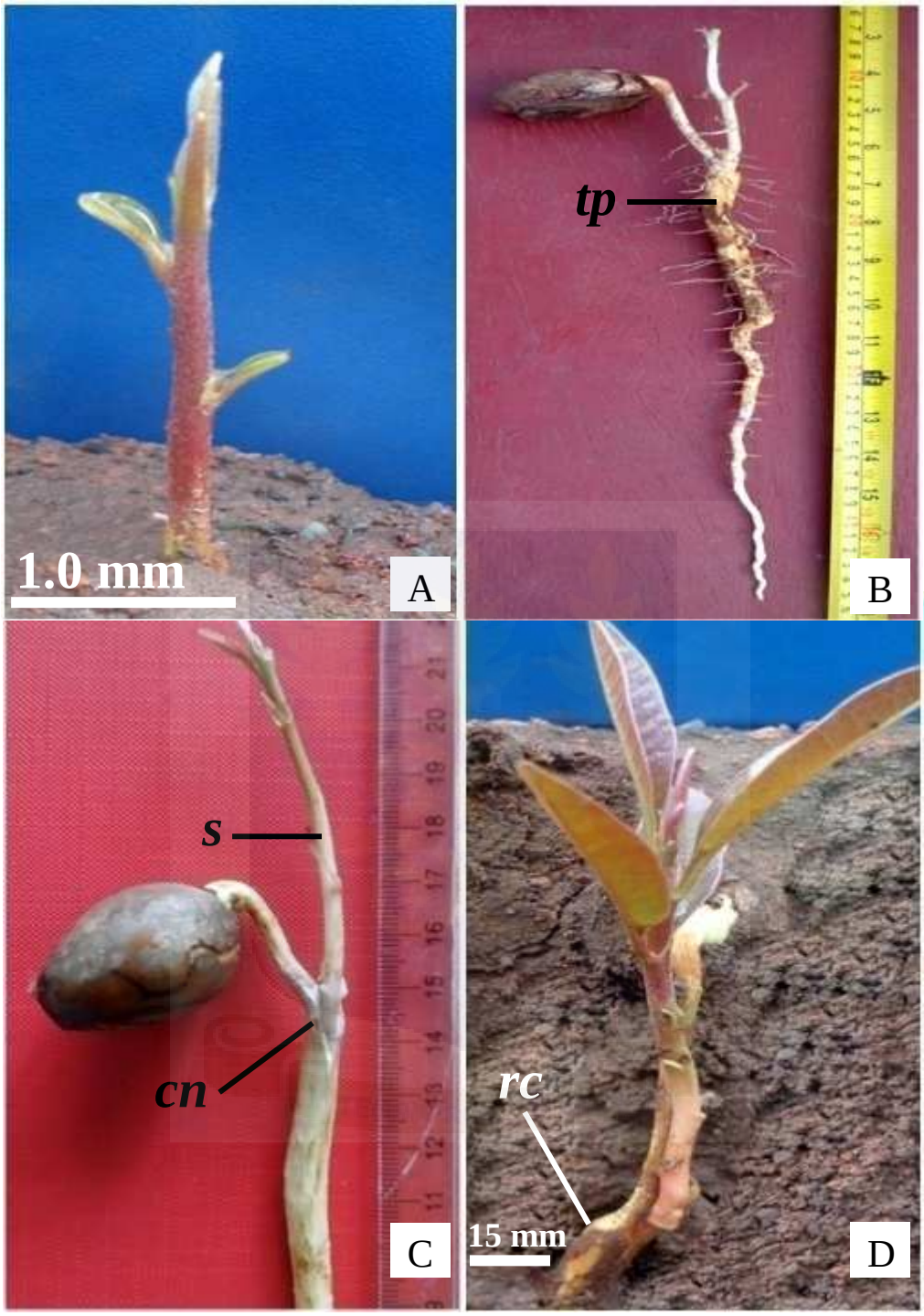

Fig. 4.11. Morphological features of *Vitellaria paradoxa* seedlings; A, Emerged seedling showing shoot above soil level; B, Seedling showing a 29 cm long taproot (*tp*); C, Seedling showing cotyledonary node (*cn*) and entire shoot (*s*); D, Seedling showing root crown (*rc*) that is buried 6.5 cm deep

4.3.3. Effects of deshelling of seeds on the germination and growth of *Vitellaria paradoxa* seedlings

Germination was observed in both the intact and deshelled seeds at the first 5 days after sowing. The germination percentage of the seeds varied non-significantly from 89.87 % for the intact, or control seeds to 94.76 % for the deshelled seeds (Table 4.6). Also, the mean germination time (11 days) for the intact seeds was not significantly different from that of the deshelled seeds (10 days).

The last seedling, produced from an intact seed, emerged at 145 DAS with 2 shoots (Fig. 4.12). However, the percentage of seedlings that emerged from the deshelled seeds was significantly higher (94.67 %) than that for seedlings produced from the intact seeds (82.99 %). The emergence index of seedlings produced from the deshelled seeds (57 days) was significantly ($P < 0.05$) shorter than that of the intact seeds (75 days). Similarly, the corresponding emergence rate index of seedlings of the deshelled seeds (60 days) was significantly shorter than that of the seedlings of the control seeds (90 days).

Table 4.6. Effects of deshelling of seeds on germination and emergence parameters of *Vitellaria paradoxa* seedlings

Seed type	Germination percentage	Mean germ. Time (days)	Emergence percentage	Emergence index (days)	Emergence rate index (days)
Intact	89.87a	10.92a	82.99b	74.68b	90.00b
Deshelled	94.67a	9.57a	94.67a	56.54a	59.70a

Means in the same column followed by different letters are significantly different ($P < 0.05$)

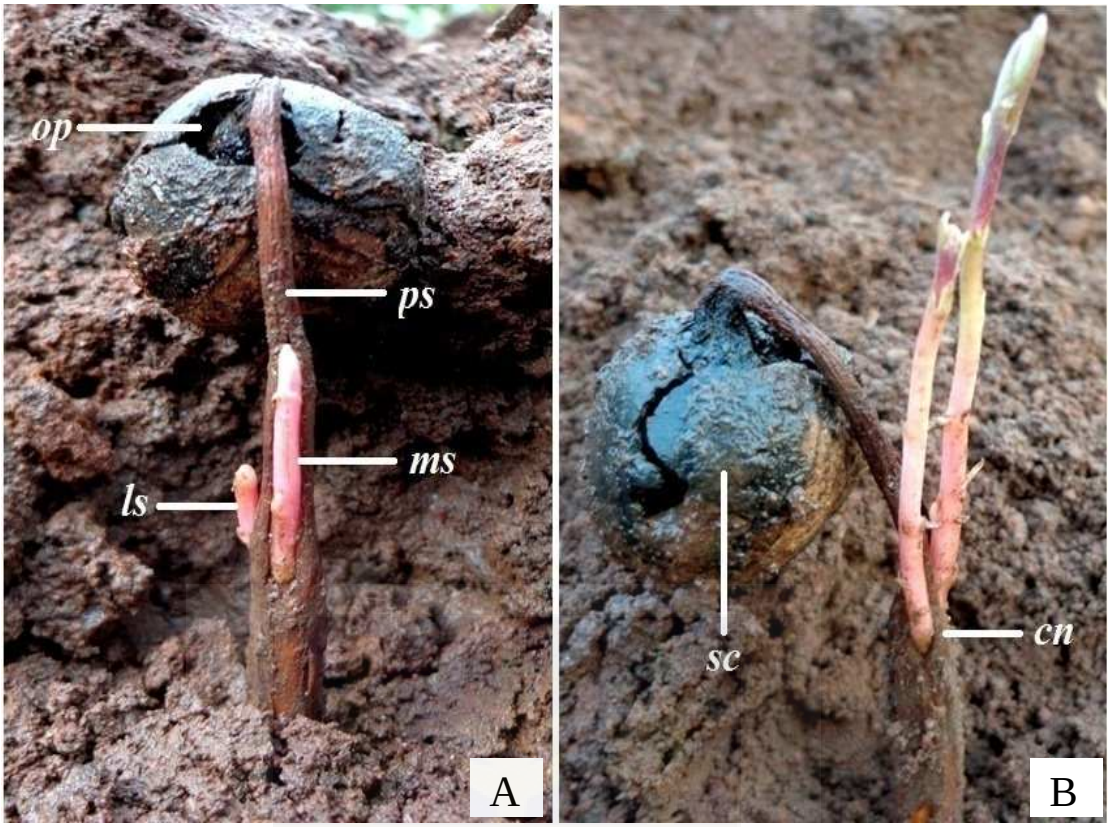

Fig. 4.12. Emergence of a trapped *Vitellaria paradoxa* seedling; A, Lateral shoot (*ls*) developing from the main shoot (*ms*) which was trapped by the pseudoradicle (*ps*); B, Cotyledonary node (*cn*) with two shoots; *op*, operculum; *sc*, seedcoat

At 150 days after sowing, seedlings produced by the deshelled seeds produced a mean stem diameter of 0.41 cm which was non-significantly larger than that produced by seedlings of the intact seeds (Table 4.7). The mean height of seedlings produced by the deshelled seeds was 5.79 cm whilst that of the seedlings of the intact seeds was 5.04 cm. A significant difference in the height of the seedlings, therefore, occurred at 150 DAS. In contrast to seedling height, the mean numbers of leaves produced by the seedlings (6.62 and 5.55 for the seedlings from deshelled and intact seeds respectively) were statistically similar to each other. Similarly, no significant difference existed between the leaf areas of the seedlings. However, mean root crown

diameter of the seedlings produced from deshelled seeds (0.78 cm) was significantly wider than that of seedlings of the intact seeds (0.42 cm).

Table 4.7. Effect of deshelling of seeds on the growth of *Vitellaria paradoxa* seedlings at 150 days after sowing

Seed	Stem diameter (cm)	Height (cm)	Number of leaves	Leaf area (cm^2)	Root collar diameter (cm)
Intact	0.35a	5.04b	5.55a	38.04a	0.42b
Deshelled	0.41a	5.79a	6.62a	51.91a	0.78a

Means in the same column followed by the same letters are not significantly different ($P < 0.05$)

Stem diameter of seedlings produced from the deshelled seeds increased more than two times at 240 DAS as compared with that recorded at 150 DAS. Consequently, the mean stem diameter of seedlings of deshelled seeds (1.12 cm) was significantly wider than that of seedlings of intact seeds (0.54 cm) (Table 4.8). Seedlings produced by the deshelled seeds had a mean height of 11.10 cm but it was non-significantly different from that recorded for seedlings of the control seeds (10.35 cm). Similarly, the mean numbers of the leaves produced by the seedlings were non-significantly different from each other (Table 4.8). In contrast, both leaf area and root collar diameter were significantly different. The leaf area of seedlings produced by the deshelled seeds (194.51 cm^2) was significantly wider than that of seedlings of the control seeds (138.15 cm^2). Also, the root collar diameter of seedlings of deshelled seeds (2.64 cm) was significantly greater than that of the seedlings of the intact seeds (1.58 cm).

Table 4.8. Effect of deshelling of seeds on the growth of *Vitellaria paradoxa* seedlings at 240 days after sowing

Seed	Stem diameter (cm)	Height (cm)	Number of leaves	Leaf area (cm^2)	Root collar diameter (cm)
Intact	0.54b	10.35a	13.01a	138.15b	1.58b
Deshelled	1.12a	11.10a	14.65a	194.51a	2.64a

Means in the same column followed by different letters are significantly different ($P < 0.05$)

Morphologically, some seedlings produced by the deshelled seeds developed 2 or more shoots above the soil level (Fig. 4.13). These seedlings still had their main growing axes (leaders) intact. Lateral shoots were produced only by the seedlings with much swollen root crowns (Fig. 4.13B). These lateral shoots were distinguished from axillary shoots by their upright growth pattern. Digging up the soil around the seedlings and examining them closely revealed that the lateral shoots were produced from two different sides, one of which was located belowground. The belowground lateral shoots were produced directly from the top of the root collar (Fig. 4.13B). These shoots appeared 2 to 4 weeks after the main axes had emerged.

The second group of lateral shoots arose from the main growing axes on top of the root collar just at or above the soil level (Fig. 4.13C and D). Such growing axes from which they arose also appear swollen right from the root collar up to the soil level (Fig. 4.13C). These shoots appeared 6 to 8 weeks after the emergence of the leaders. These lateral shoots arising directing from the leaders, and not from the root crowns, grew so quickly that irrespective of their time of appearance, they soon became the tallest shoots in just 2–3 weeks suggesting that they may be an excellent source of scions for grafting or shoot tips for *in vitro* culture (Fig. 4.13C and D).

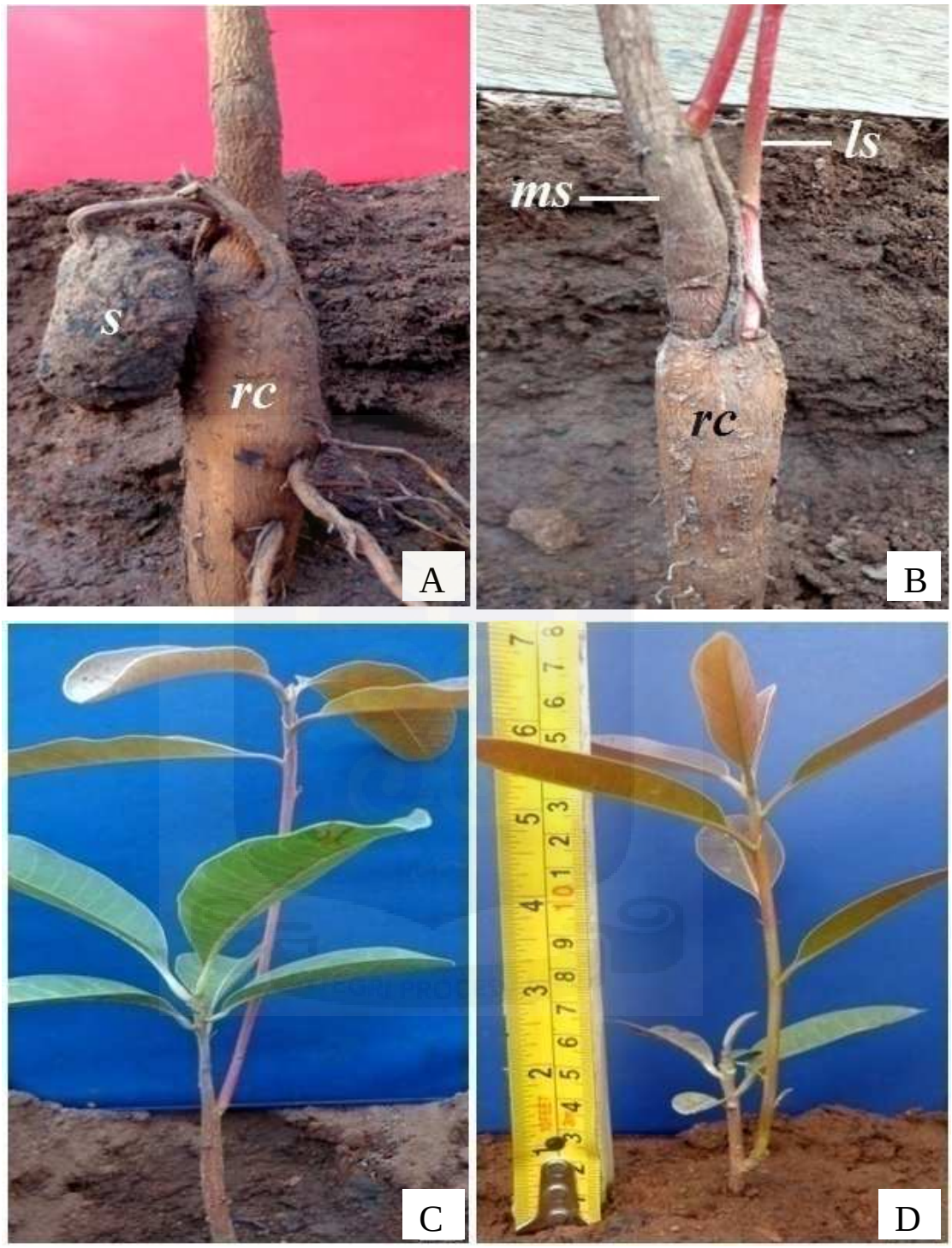

Fig.4.13. Tuberous root crown of *Vitellaria* seedlings; A, Seedling produced by a deshelled seed (*s*) showing a tuberous root crown (*rc*); B, Lateral shoot (*ls*) growing from the root crown; C, Lateral shoot growing from the main axis below the soil level; D, A 15 day-old lateral shoot developing from a 240-day old main shoot above the soil level

Apical growth of the seedlings occurred in 2 contrasting forms. In the commoner form, which was observed in 99 % of seedlings of the intact seeds, the shoot apical meristem (SAM) continuously grew upwards with or without producing axillary shoots or branches (monopodial growth). Those seedlings without branches were upright (Fig 4.14A) whilst those with one or more branches had their SAMs bent towards the direction of the branches (Fig. 4.14B).

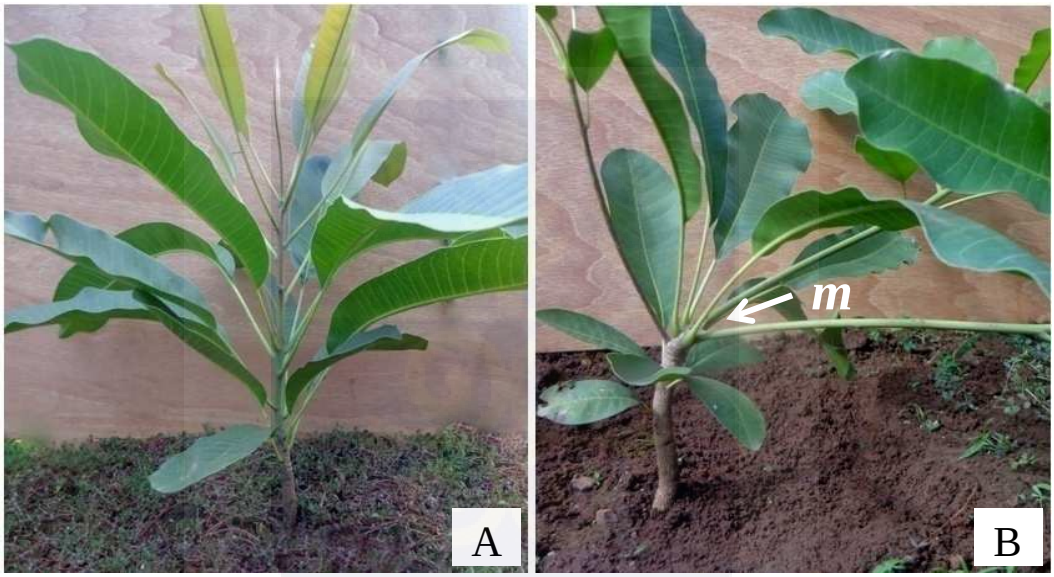

Fig. 4.14. *Vitellaria paradoxa* seedlings showing monopodial growth; A, Seedling without branches; B, Seedling with 3 branches and a bent shoot apical meristem (*m*)

In the second growth or branching pattern, which occurred in only seedlings produced by the deshelled seeds and accounted for 30 % of their population, a lateral shoot initially emerged from the tip of the shoot apical meristem and grew upwards at an angle less than 30° (Fig. 4.15A). The lateral shoot increased in length up to 12–19 cm before another one emerged from it and also grew upwards. At the time the other shoot emerged from it, the lower SAM from which it had emerged had also started producing new growth (Fig. 4.15 B).

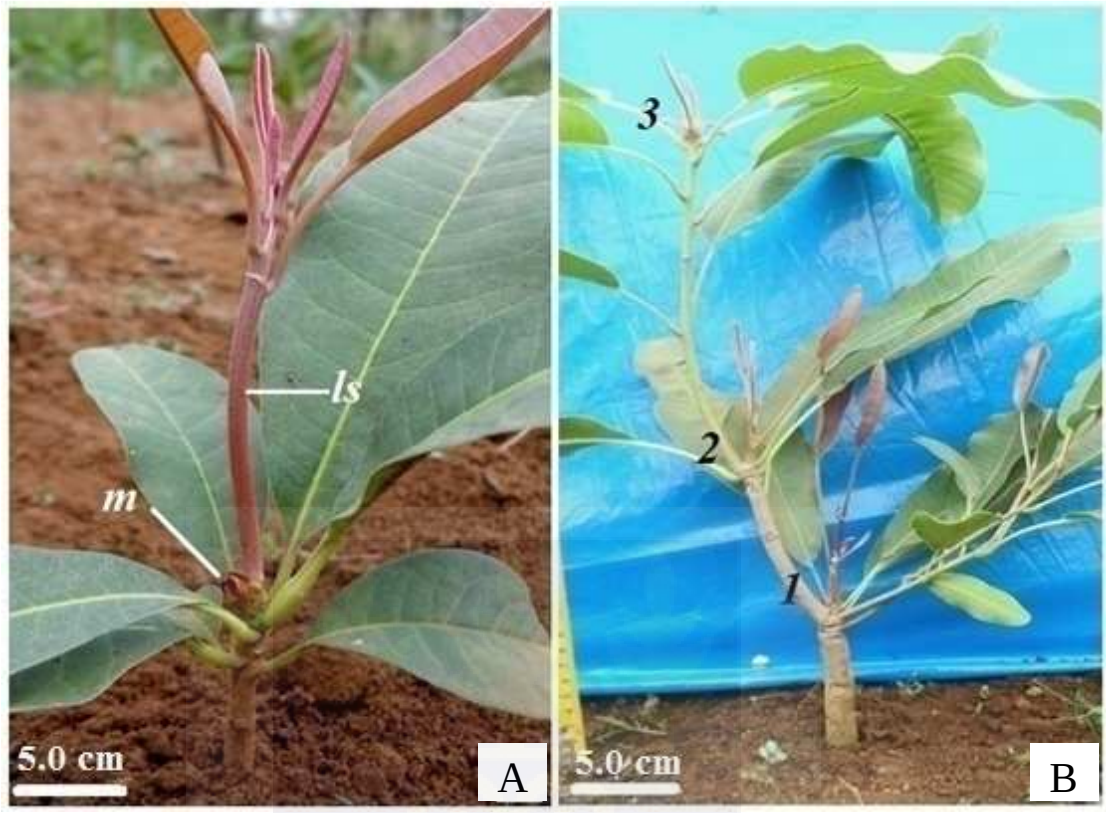

Fig. 4.15. *Vitellaria paradoxa* seedlings with two (A) and three (B) apical growing points; Lateral shoot (*ls*) produced on top of the shoot apical meristem (*m*); *1, 2* and *3* are three apical growing points

4.4. Discussion

4.4.1. Development of *Vitellaria paradoxa* seedlings

Germination or sprouting of *Vitellaria* seeds commenced when the pseudoradicles protruded through the seedcoats via the opercula by pushing against and rupturing the overlying operculum caps. This observation is in contrast to germination in most dicotyledonous plants in which the radicle protrudes the seedcoat through the micropyle. Pérez, (2009) and Gong *et al*. (2005) observed that radicles or pseudoradicles of monocotyledonous plants commonly protrude from the seed via the opercula; thus, operculum protrusion in *V. paradoxa* (a eudicot) makes its

germination unique. The large size of the pseudoradicle relative to that of the micropyle most likely precludes micropylar protrusion.

Proximal protrusion (pseudoradicles appearing at the proximal or micropylar end of the seeds) represented 92 % of the protrusions with the remainder representing pseudoradicles protruding from other parts of the seedcoat. The embryos which swell to form the pseudoradicles are occasionally located in any other parts of seed besides the micropylar ends. This seed morphology explains why the pseudoradicles protruded from different parts of the seedcoat as observed in this study. This observation supports the finding of Msanga (1998) who recorded radicles of some tropical plants such as *Hopea ferrea* and *Markhamia lutea* protruding from the seedcoat via different parts other than the micropylar end in contrast to temperate plants whose radicles exclusively protrude the seedcoat from the micropyle.

The development of *V. paradoxa* seedlings occurred in 7 distinct stages namely: sprouting, pseudoradicle elongation, bulging, shoot appearance, shoot elongation, emergence and establishment. Jackson (1968) described the germination of *V. paradoxa* but never identified the distinct stages as done in this study. Ugese *et al*. (2010) outlined only 5 stages because shoot appearance and shoot elongation were considered as a single stage whilst establishment was excluded.

The pseudoradicle of a sprouted *Vitellaria* seed elongates to push the plumule and the radicle deeper into the soil. The morphology of the pseudoradicle clearly indicates that it is the fused petioles of the 2 cotyledons of the seeds and may therefore be described as the cotyledonary tube. Alabarce and Dillenburg (2012) and Burrows and

Stockey (1994) used the term cotyledonary tube to describe a similar positively geotropic structure produced by cryptogeally germinating *Araucaria angustifolia and A. bidwillii* respectively. Another term that is suggested based on observations made in this study is cotyledonary axis, which is analogous with embryonic axis.

A transverse section through the cotyledonary tube or pseudoradicle showed an outer sheath, laticiferous vessels and an inner hollow tube. Functionally, the outer sheath protects both the shoot and root apical meristems in its hollow tube. The laticiferous vessels translocate both latex sap and food reserves from the seed to the base of the bulge where the embryo develops. Germinating seeds that developed 6 laticiferous vessels produced 1 shoot each. However, germinating seeds with 7 or 8 lactiferous vessels typically produced 2 or more shoots (multiple shoots) above the soil level. The increased number of laticiferous vessels might have facilitated rapid translocation of seed reserves to the base of the pseudoradicle for use by the developing plumules or shoots which in turn caused them to develop branches.

The number of pseudoradicles per germinating seeds also varied resulting in 1 seedling (monoembryony) or 2 seedlings (polyembryony). Monoembryonic seeds that produced 1 pseudoradicle characteristically produced 1 seedling. The single seedling may emerge above the soil with 1 or more shoots (multiple shoots). Multiple shoots were most likely produced by precocious branching of the plumules which resulted in 2 or more shoots appearing via the cotyledonary slits. Polyembryony which results in the production of 2 or more seedlings from 1 seed has already been reported in an Indian Sapotaceae *Madhuca indica* (Verma *et al*., 2009). It may, thus, account for the

production of 2 seedlings from 1 seed as observed in this study. Multiple seedlings can readily be separated and replanted just as monoembryonic seedlings.

Vitellaria paradoxa produces cryptogeal seedlings (Benti, 2009) but this seedling morphology remains unreported in any other sapotaceous species. Ibarra-Manríquez *et al*. (2001) reported that seedling traits are evolutionary conservative reflecting phylogenetic relationships. Therefore, *Vitellaria* might have evolved cryptogeal germination in response to bushfires occurring widely in the Shea Belt. Jackson (1968) observed that when *Vitellaria* seedlings push their plumules and radicles into the soil via the elongating fused cotyledonary petioles, they safely bury their root crowns in soil where they are protected against both bushfires and heat during the long dry season. Accordingly, Jackson (1974) described cryptogeal germination as plumule burying. Another benefit of cryptogeal germination is that it enables the seedlings to resprout quickly after grazing and fires (Clarkson and Clifford, 1987).

In this study, seedlings of *V. paradoxa* were either cryptocotylar or phanerocotylar reserve types depending on cotyledon morphology. Seeds whose cotyledonary raphes run parallel to their embryos produced phanerocotylar seedlings, whereas those whose cotyledonary raphes were perpendicular to their embryos produced cryptocotylar seedlings. Sapotaceous species produce both cryptocotylar and phanerocotylar seedlings. For example, Cruz (2005) observed phanerocotylar seedlings in *Pouteria pachycarpa* whilst Mundhra and Paria (2009) described cryptocotylar seedlings in *Madhuca indica*. Essig (1987) reported that within any given genus, different subgenera could be distinguished by seedling morphologies. Thus, the production of

both phanerocotylar and cryptocotylar seedlings may be useful for taxonomic classification within the genus *Vitellaria*.

4.4.2. Seed size and development of *Vitellaria paradoxa* seedlings

The germination of *Vitellaria* seeds and the subsequent morphology of the seedlings were significantly influenced by sizes of the seeds sown. In contrast, Ugese *et al*. (2008b) observed lower emergence percentage when small-sized seeds were sown as compared with large ones. The high germination percentage of all the seeds used in this study could probably be due to their freshness. Jøker (2000) reported that *Vitellaria* seeds have to be sown fresh because they have a very high germination percentage at that state but it decreases with loss of moisture.

The time to sprouting or germination of large seeds was significantly ($P < 0.05$) earlier (7 days) than those of medium and small seeds. Ugese *et al*. (2010) had earlier reported that *V. paradoxa* seeds often sprout within 7 days when sown. Large seeds germinated earlier most probably because their developing embryos have more seed reserves, and their widest area could have also enabled them to imbibe more moisture rapidly. Subsequently, the pseudoradicles of the seedlings produced by large seeds elongated faster producing bulges quicker than those of medium and small seeds. On the contrary, germination was delayed in small seeds, an observation which had also been reported by Ugese *et al*. (2007). The delayed germination may be attributed to little food reserves in the small seeds.

The interval between the appearance of the shoot on the pseudoradicle and seedling emergence denoted by SE (shoot elongation) represented the single longest period in

the skotomorphogenic growth of *Vitellaria* seedlings. Shoots of seedlings produced from small seeds took the longest period to elongate fully. This delayed shoot elongation was most likely caused by obstruction of the shoots by the pseudoradicles or by the seeds or by both. Due to the presence of more reserves, both medium and large seeds produced seedlings with more vigorous shoots, which were less obstructed. Without giving any methods, Ugese *et al*. (2010) suggested that *V. paradoxa* seedlings could emerge quickly by manipulating them at any of the stages between bulging and emergence. One of such methods is turning the seed into a different direction any time the shoots appear ventral on the pseudoradicle.

The emergence index of seedlings produced by large seeds was significantly shorter than that of seedlings of small seeds. The faster emergence could be due to the presence of more food reserves, which were then mobilized for growth. According to Ugese *et al*. (2010), emergence of *V. paradoxa* seedlings ranged from 51 to 79 days after sowing. In this study, seedling emergence ranged from 61 to 75 days depending on the seed size. Contrastingly, Yidana (2004) reported that *V. paradoxa* seedlings emerge as early as 28 days after sowing. The differences in seedling emergence indices may be due to differences in their genetic make-up.

Large seeds produced seedlings which exhibited vigorous growth in terms of height of shoots and diameters of the root collars and shoots. The vigorous growth observed in shoots developing from these seedlings may again be attributed to the large amount of food reserves available for the developing shoots. These seedlings also developed shorter taproots suggesting that they could easily be raised in nursery containers. Contrastingly, National Research Council (2006) reported that the containerization

and subsequent transplanting of *V. paradoxa* seedlings are difficult because they develop long taproots which quickly outgrow the containers.

However, seedlings produced from small and medium seeds developed longer taproots which usually adapt them well to their ecological conditions. Optimal partitioning theory predicts that plants allocate biomass preferentially to harness resources that are most limiting to growth (Kobe *et al*., 2010). In the Shea Belt, the most-limiting factor to plant growth is insufficient moisture which is usually accompanied by widespread bushfires (Jackson, 1974). The longer taproots of seedlings of small and medium seeds may enable them to reach moisture deep into the soil than those produced by large seeds. Also, the longer pseudoradicles of these seedlings pushed their shoot meristems located in the root crown deeper into the soil. Thus, seedlings produced from small and medium seeds are better equipped with the ability to resprout than those of large seeds.

4.4.3. Growth and morphology of *Vitellaria paradoxa* seedlings

Although seedcoat or testa plays a protective role in seeds, it imposes dormancy, thereby inhibiting germination in most species (Debeaujon *et al*., 2000). Thus, the effect of seedcoat on germination and seedling growth of *V. paradoxa* was studied by removal of the testa. Deshelling did not have any significant ($P > 0.05$) effect on mean germination time and germination percentage. However, it significantly ($P < 0.05$) influenced emergence index, emergence percentage and emergence rate index.

The non-significant difference in germination between the deshelled and intact seeds suggests that in *V. paradoxa*, the seedcoat does not impose dormancy on the embryo.

Ugese *et al*. (2005) earlier concluded that any treatment aimed at breaking dormancy or achieving quicker seedling emergence should not be targeted at the seedcoat. Jøker (2000) emphasized that *Vitellaria* seeds require no pre-treatment besides extraction from the fruit because they are non-dormant.

On the contrary, the removal of the shell allowed the cotyledons to swell quickly into a pseudoradicle which in turn elongated rapidly. This rapid pseudoradicle elongation might have accounted for the significantly higher emergence percentage, and the smaller emergence index and emergence rate index of seedlings produced by the deshelled seeds than those produced by the intact seeds. Smaller emergence and emergence rate indices indicate faster and synchronous seedling emergence (Ugese *et al*., 2011). The emergence of seedlings from the deshelled seeds suggests a highly synchronized seedling development. Therefore, removal of the shell of *V. paradoxa* seed has the potential of producing more uniform seedlings in the nursery.

In this study, the last seedling which emerged 145 days after sowing (DAS) had its main axis obstructed by the pseudoradicle and this obstruction caused the seedling to produce 2 shoots. According to Jøker (2000), seedlings of the subspecies *V. nilotica* emerged faster than those of *V. paradoxa* in which emergence could even occur 150 DAS. The delayed emergence of *V. paradoxa* seedlings is caused by the shoots being obstructed from elongating freely through the soil, usually by the pseudoradicles and the seeds or by both. In non-obstructed seedlings, emergence never exceeded 70 DAS. One of the most likely factors explaining why seedlings produced by the deshelled seeds were less obstructed was that their pseudoradicles and seeds withered and shrank more rapidly. This rapid withering and shrinking of seed remnants might also

explain why the emergence percentage of these seedlings was significantly higher than that of the seedlings produced by the intact seeds.

Deshelling of the seeds did not produce any significant effect on height and number of leaves produced by the seedlings. In contrast, however, the mean stem and root crown diameters as well as the leaf area were significantly ($P < 0.05$) influenced by the removal of the seedcoat. The rate of leaf production is always proportional to apical growth in seedlings because leaves are produced from nodes which result in increased seedling height. Thus, the non-significant difference in seedling height also translated into the non-statistical difference in mean number of leaves produced by both seedling types. Asante *et al*. (2012) who earlier recorded a mean highest number of 4.6 leaves produced by 24-week-old *V. paradoxa* seedlings attributed that observation to their cryptogeal morphology. Ugese (2010) observed a positive, significant correlation between leaf size and stem diameter of tamarind (*Tamarindus indica* L.) seedlings. The significantly wider leaves of the seedlings of deshelled seeds produced more photosynthates which they eventually used to develop bigger stems.

Cryptogeally germinating seeds have evolved a means of transferring the reserves in their surface-borne seeds and other aboveground parts onto underground sinks which appear swollen throughout their sapling stage and enable them to persist in the seedling bank of the soil (Dillenburg *et al*., 2010). Therefore, photosynthates mobilized by the seedlings were subsequently translocated below ground. The wider root crowns of seedlings produced by the deshelled seeds could have been due to the presence of more photosynthates which were produced using their wider leaves.

Only seedlings produced by deshelled seeds which developed the larger root crown diameters produced both below- and aboveground lateral shoots. Diarrassouba *et al*. (2009) and Nikiema and Umali (2007) reported that *V. paradoxa* produces plagiotropic branches 4–7 years after sowing. By their orthotropic growth, lateral shoots observed in this study were clearly distinguished from branches. The production of 2 or more shoots which gives rise to several growing axes and branches numbering as many as 5 per seedling was considered phenomenal. Lovett and Haq (2000) explained that the current genetic make-up of *Vitellaria* reflects a millennium of anthropic selection due to its association with human habitation. Thus, semi-domestication is most likely being manifested by *V. paradoxa* seedlings which are less than one year old and yet produced 2 or more growing points or axillary shoots.

4.5. Conclusion

The development of a *Vitellaria paradoxa* seedling occurs in 7 distinct stages termed: sprouting, elongation of the pseudoradicle, bulging, shoot appearance, shoot elongation, emergence and establishment with shoot elongation below ground being the longest. At shoot appearance stage, turning the pseudoradicles with the seeds into another direction may shorten the duration to emergence. Large seeds produced seedlings with vigorous aboveground growth and with shorter taproots. Medium and small seeds produced seedlings with longer pseudoradicles and taproots. Where seeds are to be planted *in situ*, medium seeds may be used whilst large seeds may be preferable for nursery establishment. Deshelling of seeds prevented seedling trapping and therefore resulted in higher germination percentage, shorter emergence index and a more synchronous seedling development.

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CHAPTER 5

5.0 *In vitro* propagation of *Vitellaria paradoxa*

5.1. Introduction

Although *V. paradoxa* is amenable to both sexual and asexual methods of reproduction, the long juvenile growth period of saplings, stem and root cuttings, and grafted seedlings makes *ex vitro* propagation methods agronomically unattractive (Yeboah *et al*., 2009). Moreover, the recalcitrant seeds are only available during April to September and lose viability easily through desiccation (Danthu *et al*., 2000). In addition, seeds germinate non-synchronously making it difficult to produce uniform seedlings on large-scale basis. As has already been reported for other sapotaceous species such as *Argania spinosa* (Nouaim *et al*., 2002) and *Pouteria lacuma* (Padilla *et al*., 2006), *in vitro* propagation may be an effective option for propagating this economically important species

Several researchers including CRIG (2012) and Fotso *et al*. (2008) have attempted *in vitro* regeneration of *V. paradoxa* because every living cell is totipotent, but success rate has thus far been low. The low success rate may be attributed to saponins and latex sap which are abundantly exuded from any living part of the plant. These exudates reduce the success rate of grafting (Masters, 2002) and could be a source of *in vitro* contamination. For other reasons precisely unknown, somatic embryos of *Vitellaria* are difficult to transform into plantlets (Fotso *et al*., 2008) or the resulting plantlets are easily lost during post-flask management (CRIG, 2012).

Despite the losses of *in vitro* plantlets, Adu-Gyamfi *et al*. (2012) successfully regenerated *V. paradoxa* plantlets by somatic embryogenesis using immature cotyledon explants. The cotyledon explants were cultured on Murashige and Skoog

(MS) (1962) basal medium supplemented with 30.0 g/l sucrose, 2.4 g/l phytagel and 5 different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) (0–0.5 mg/l) to induce embryogenic calli. The embryogenic calli developed into somatic embryos when transferred onto a hormone-free MS medium amended as described earlier. In addition to non-synchronous development, the somatic embryos germinated poorly (15 %). Lovett and Haq (2013) also successfully regenerated *Vitellaria* using shoot tips explanted from 1 to 2-month-old seedlings and cultured on half- and quarter-strength MS medium supplemented with 6-benzyladenine (BA) and NAA. However, transfer of the regenerated shoots into a rooting medium resulted in less than 30 % successful root induction. Therefore, other protocols that may result in high success rate leading to the production of plantlets in large quantities are yet to be developed.

Latex-related contaminations reduce the success of many *in vitro* techniques used for propagating sapotaceous species. Being laticiferous species, the Sapotaceae produce the latex sap as a secondary metabolite when they are developing making its total elimination from the growth media difficult (Bhore and Preveena, 2011). Repeated subculturing to minimize latex contamination not only increases cost of production but also leads to losses of plantlets. Thus, one of the most feasible options for successful *in vitro* regeneration of *Vitellaria* would be to identify and to excise any part of the seed or seedling that contains little or no latex for culture. The major objective of this study was therefore to develop an *in vitro* protocol for *V. paradoxa*. The specific objectives were to

i. propagate *V. paradoxa* using whole seeds as explants
ii. regenerate *V. paradoxa* plantlets using embryonic axis explants
iii. regenerate *V. paradoxa* using rudimentary shoots of *in vivo* seedlings.

5.2. Materials and methods

5.2.1. Collection of *Vitellaria paradoxa* fruits

Mature fruits of *V. paradoxa* collected from farmlands and fallows at Tanina and Ga in the Wa West District of the Upper West Region of Ghana were used for this study (Section 3.2.1).

5.2.2. *In vitro* culture of intact seeds

Fresh fruits were depulped manually to obtain seeds. Eighty (80) seeds were selected and thoroughly washed in distilled water and air-dried for 24 hours. They were then sterilized by immersing 0.2 % mercuric chloride ($HgCl_2$) for 2 minutes followed by rinsing with 3 changes of sterile distilled water and cultured on 60 ml Murashige and Skoog (1962) basal medium in honey jars. The MS medium was prepared from stock solutions and amended with 30.0 g/l sucrose, 100.0 mg/l myo-inositol, 100.0 mg/l copper sulphate ($CuSO_4$), 100.0 mg/l activated charcoal and 1.0, 2.0, 3.0 or 4.0 mg/l BAP. The pH of the medium was adjusted to 5.8 using 1.0 M KOH before the addition of 3.0 g/l phytagel and autoclaving at 121 ℃ for 15 minutes at 15 psi. The cultured explants were incubated in the growth room at a temperature of 25 ± 2 ℃ under 16-hour photoperiod with light provided by fluorescent tubes at an intensity of 3000 lux. Completely randomized design was used with 20 seeds per each of the 4 different concentrations of BAP. The number of days to sprouting, sprouting percentage, days to seedling emergence, seedling height and number of leaves were recorded. Seeds were considered sprouted when their pseudoradicles became visible.

5.2.3. *In vitro* culture of deshelled seeds

Eighty (80) freshly extracted seeds were deshelled by pressing the seeds in between pliers to rupture the shell (testa) at the dorsal side. A knife was then used to remove the remaining parts of the shell. The deshelled seeds were thoroughly washed and rinsed with 4 changes in distilled water and air-dried for 12 hours. They were soaked in 100.0 mg/l ascorbic acid solution for 3 hours to remove phenolic compounds in them and then sterilized using 0.2 % mercuric chloride ($HgCl_2$) for 90 seconds and cultured on MS medium prepared as described in Section 5.2.2. The growth room conditions in which the cultured seeds were incubated, the experimental design and data collection were also the same as outlined in Section 5.2.2.

5.2.4. Identification and culture of embryonic axes

Eighty (80) freshly extracted seeds prepared as already described (Section 5.2.3) were soaked in distilled water for 6 hours. The deshelled seeds were again soaked in 1.0 % TTC solution to identify their embryos (see Section 3.3). The stained embryo spots or axes were excised using a 1.0 cm diameter cork borer, trimmed to 0.8 cm long and then soaked in a 100.0 mg/l solution of ascorbic acid for 3 hours to remove any phenolic compounds. They were immersed in 0.2 % mercuric chloride ($HgCl_2$) for 60 seconds and then rinsed with 4 changes of sterile distilled water. After the sterilization, the embryonic axes were cultured in honey jars containing 60 ml MS medium supplemented with 30.0 g/l sucrose, 100.0 mg/l myo-inositol, 100.0 mg/l copper sulphate ($CuSO_4$), 100.0 mg/l activated charcoal, 3.0 g/l phytagel and 1.0, 2.0, 3.0 or 4.0 mg/l BAP prepared as described earlier and incubated under the same growth room conditions (see Section 5.2.2). The experimental design and data collection were the same as described in Section 5.2.2.

5.2.5. *In vitro* culture of rudimentary shoots

Freshly extracted seeds were washed using tap water and air-dried for 6 hours. The seeds were treated with Hercule^{R} 50 SC (IPROCHEM Co. Ltd, Shenzhen, China) against termites and then sown 2 cm deep with the hilum down in polyethylene pots filled with a mixture of compost and sawdust in the ratio 5:1. The polyethylene pots with the sown seeds were placed in a plant barn and watered every other day. The germinated seeds were uprooted after 2–3 weeks and the bulged portions of the pseudoradicles containing the rudimentary shoots were excised using forceps. The excised portions measuring 1.5 cm each were sterilized by washing thoroughly under tap water and thereafter soaked in distilled water for 1 hour to remove any latex. They were immersed in 0.2 % mercuric chloride ($HgCl_2$) for 90 seconds and rinsed with 3 changes of sterile distilled water. They were then dissected to remove the rudimentary shoots which were cultured in honey jars containing 40 ml MS basal medium supplemented with 30.0 g/l sucrose, 100.0 mg/l myo-inositol, 100.0 mg/l copper sulphate and 1.0, 2.0, 3.0 or 4.0 mg/l BAP and 0.0, 0.1, 0.2 or 0.4 mg/l NAA. The culture medium was prepared as described in Section 5.2.2. The cultured explants were incubated in the growth room under the same conditions as described in Section 5.2.2. The factorial experimental design comprising 2 factors was used. The duration to response of explants to culture, height of developing shoots, and the number of leaves, root and shoots produced per explant were recorded. Response of explants to culture was determined by change in colour (pink to light green). Changes in colour of the cultured explants were described using HTML Colour Chart (http://www.html-color-names.com/color-chart.php). Observations were made at 5-day intervals beginning from the day of inoculation.

5.2.6. Data analysis

Data were subjected to analysis of variance using Genstat statistical package (9th Edition). Means were separated where appropriate using the least significant difference (LSD) test.

5.3. Results

5.3.1. *In vitro* germination of intact seeds

Between 5 and 10 days after culture, almost all the seeds showed signs of germination because their seedcoats had ruptured visibly. Thereafter, they exuded phenolics excessively into the culture medium, which subsequently hindered the germination process. Consequently, all the cultured seeds died after 50 days of culture (Fig. 5.1).

Fig. 5.1. Intact *Vitellaria paradoxa* seed cultured on MS basal medium supplemented with 2.0 mg/l BAP showing visible seedcoat rupture (arrowed) 10 days after culture

5.3.2. *In vitro* germination of deshelled seeds

In contrast to intact seeds, deshelled seeds sprouted within 7 days of culture on BAP amended medium. The seeds sprouted when their cotyledons swelled and elongated into pseudoradicles at the proximal ends (Fig. 5.2). However, the number of seeds that sprouted varied depending on the concentration of BAP in the culture medium. The duration to sprouting significantly delayed as the concentration of the BAP increased suggesting that higher BAP concentration may be detrimental to sprouting (Fig. 5.3A). Also, the percentage of seeds that sprouted decreased as the concentration of the BAP in the medium increased. Eighty percent (80 %) of the deshelled seeds cultured on 1.0 mg/l BAP supplemented medium sprouted, but the sprouting decreased to 50 % at 3.0 or 4.0 mg/l BAP. However, no significant difference existed in the percentage sprouting (Fig. 5.3B). Due to intense phenolic exudation, the sprouted seeds could not develop into plantlets after 150 days of incubation.

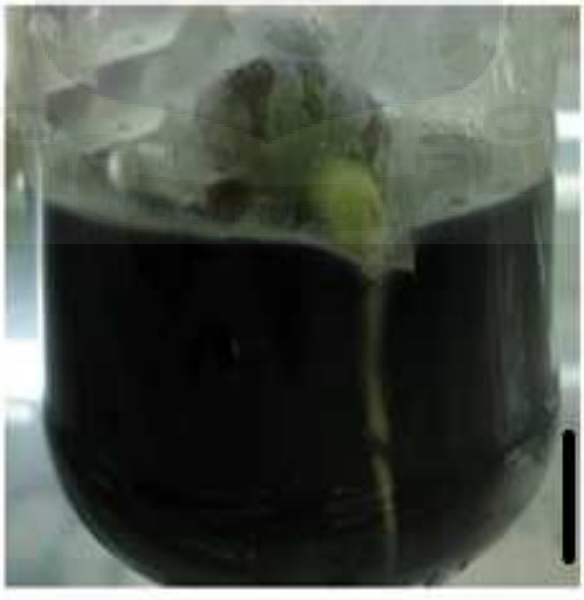

Fig. 5.2. Sprouted *Vitellaria paradoxa* seed cultured on MS basal medium amended with 1.0 mg/l BAP after 35 days of culture; Bar = 1.0 cm

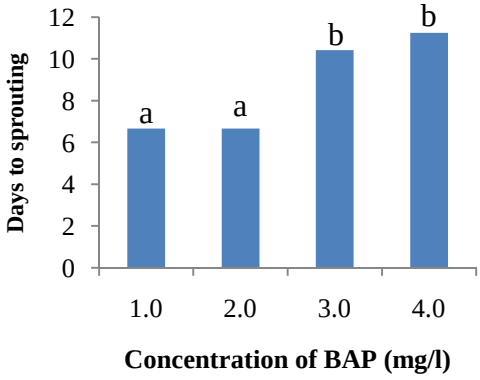

Fig. 5.3A

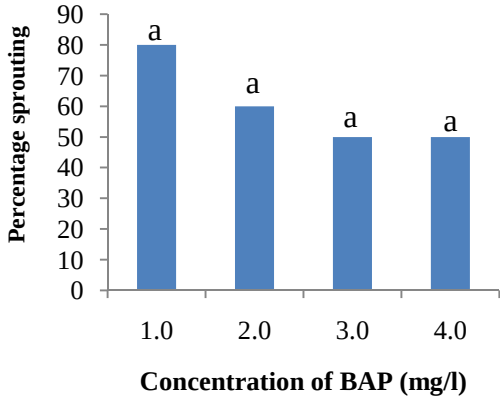

Fig. 5.3B

Fig. 5.3. Days to sprouting (A) and percentage sprouting (B) of deshelled *Vitellaria paradoxa* seeds cultured on MS basal medium supplemented with 1.0–4.0 mg/l BAP; Bars with the same letters are not significantly different ($P < 0.05$)

5.3.3. Response of embryonic axes to *in vitro* culture

Excised TTC stained embryonic axes cultured on MS medium supplemented with BAP swelled and developed pseudoradicles between 7 and 12 days after culture (Fig. 5.4). Sprouting was significantly earlier (6 days) on the medium supplemented with 1.0 mg/l BAP than on the other concentrations. On the medium with 4.0 mg/l BAP, embryonic axis explants took approximately twice the time used by those cultured on 1.0 mg/l BAP to sprout (Fig. 5.5A). Latex or phenolics were not exuded in the culture medium as compared with the culture of deshelled seeds where whitish exudates accumulated on the surface of the growth medium.

The percentage sprouting also varied depending on the concentration of BAP in the culture medium. It was significantly higher (90 %) on the medium with 2.0 mg/l BAP and lower (50 %) on the medium with 4.0 mg/l BAP (Fig. 5.5B). After 70 days of culture, the explants became brown and eventually wilted without any sign of emergence of plantlets.

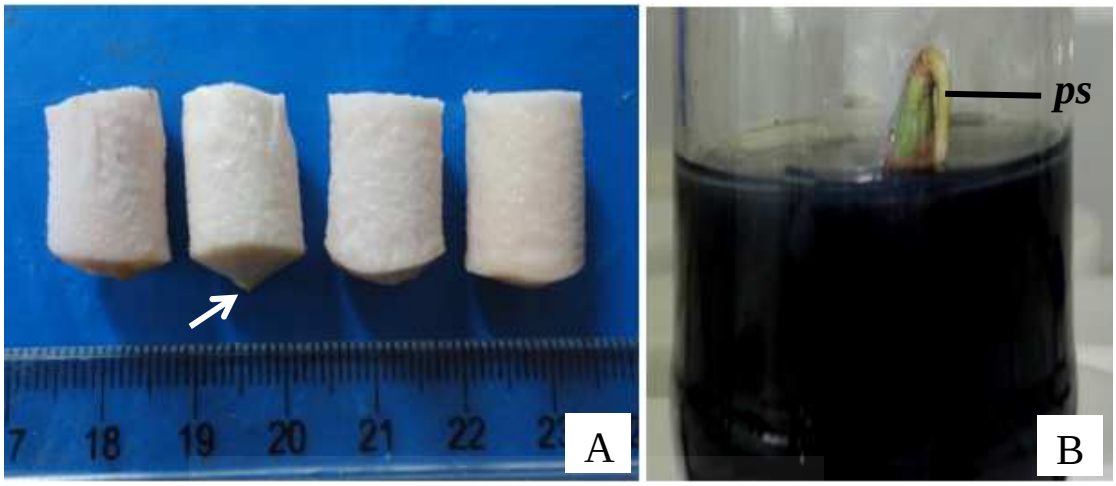

Fig. 5.4. Embryonic axis culture of *Vitellaria paradoxa*; A, Embryonic axis explants showing the embryo (arrowed); B, Sprouted embryonic axis cultured on MS basal medium amended with 1.0 mg/l BAP 10 days after culture; *ps*, pseudoradicle

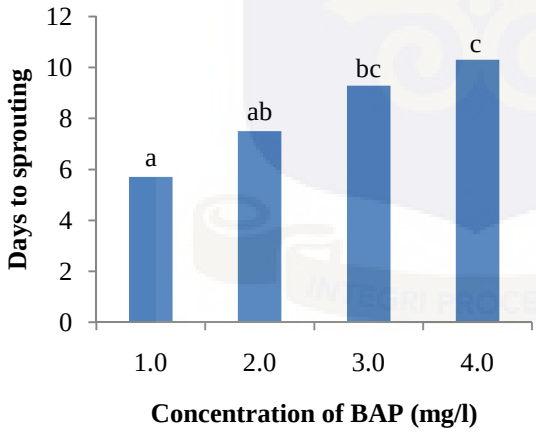

Fig. 5.5A

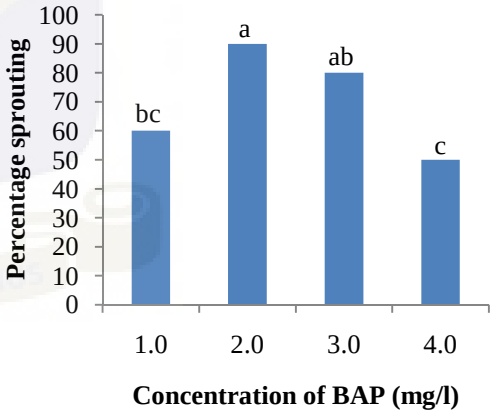

Fig. 5.5B

Fig. 5.5. Days to sprouting (A) and percentage sprouting (B) of *Vitellaria paradoxa* embryonic axes cultured on MS basal medium supplemented with 1.0–4.0 mg/l BAP; Bars with the same letters are not significantly different ($P < 0.05$)

5.3.4. *In vitro* regeneration of rudimentary shoots

Although the cultured deshelled seeds of *V. paradoxa* sprouted by producing long pseudoradicles, they never developed shoots throughout the culture period. Thus, rudimentary shoots in the bulges of the pseudoradicles from *in vivo* seedlings were excised and cultured on MS medium supplemented with BAP and NAA at different concentrations. The rudimentary shoot explants responded to culture when their scale leaves turned from pink to green within 5 to 15 days after culture (Table 5.1).

Days to response to culture were influenced by the presence of both BAP and NAA in the culture medium. An increase in BAP concentration from 1.0 mg/l enhanced the rate of response to culture conditions. All explants cultured on 1.0 mg/l BAP responded within 2 weeks independent of the NAA concentration in the culture medium. But on the medium with the highest NAA concentration (0.4 mg/l), response was delayed to 15 days compared with 8 and 10 days when NAA concentration was decreased to 0.2 or 0.1 mg/l respectively (Table 5.1).

At 2.0, 3.0 or 4.0 mg/l BAP, days to response was significantly reduced to 5 or 6 days (within a week) except when explants were cultured on a medium supplemented with 0.4 mg/l NAA. At this highest NAA concentration, response was delayed to 13 days for 2.0 mg/l BAP, 8 days for 3.0 mg/l BAP and 7 days for 4.0 mg/l BAP. Statistical analysis showed significant interactions between BAP and NAA on duration to response of explants to culture (Appendix 7.38).

Table 5.1. Effects of BAP and NAA on the response to culture, height and leaf production of rudimentary shoot explants 15 and 45 days after culture

Conc. of BAP (mg/l)	Conc. of NAA (mg/l)	Days to response to culture	Mean shoot height 15 days after culture	Mean number of leaves 15 days after culture	Mean shoot height 45 days after culture	Mean number of leaves 45 days after culture
1.0	0.0	11.67 ± 1.56de	1.33 ± 0.19cde	1.33 ± 0.41bc	1.93 ± 0.20f	2.67 ± 0.61hi
	0.1	10.00 ± 1.56cd	1.47 ± 0.19cd	1.67 ± 0.41bc	2.50 ± 0.20f	4.00 ± 0.61efg
	0.2	8.33 ± 1.56bc	1.20 ± 0.19def	2.00 ± 0.41b	1.90. ± 0.20f	3.33 ± 0.61fgh
	0.4	15.00 ± 1.56ef	0.87 ± 0.19f	1.00 ± 0.41c	1.27 ± 0.20g	2.00 ± 0.61i
2.0	0.0	5.00 ± 1.56a	1.27 ± 0.19cde	2.00 ± 0.41b	3.10 ± 0.20de	5.00 ± 0.61bcde
	0.1	**5.00 ± 1.56a**	**2.60 ± 0.19a**	**3.00 ± 0.41a**	**4.60 ± 0.20a**	5.67 ± 0.61abc
	0.2	6.67 ± 1.56ab	2.13 ± 0.19b	2.00 ± 0.41b	3.77 ± 0.20bc	**6.67 ± 0.61a**
	0.4	13.33 ± 1.56ef	1.00 ± 0.19ef	1.33 ± 0.41bc	3.00 ± 0.20e	3.00 ± 0.61ghi
3.0	0.0	5.00 ± 1.56a	1.23 ± 0.19cde	1.67 ± 0.41bc	3.53 ± 0.20bc	5.33 ± 0.61bcd
	0.1	5.00 ± 1.56a	1.43 ± 0.19cd	1.67 ± 0.41bc	3.73 ± 0.20bc	4.67 ± 0.61cde
	0.2	5.00 ± 1.56a	1.57 ± 0.19c	2.00 ± 0.41b	3.43 ± 0.20cd	5.00 ± 0.61bcde
	0.4	8.33 ± 1.56bc	0.87 ± 0.19f	1.33 ± 0.41bc	2.53 ± 0.20f	2.67 ± 0.61hi
4.0	0.0	5.00 ± 1.56a	1.43 ± 0.19cd	3.00 ± 0.41a	3.40 ± 0.20cde	5.00 ± 0.61bcde
	0.1	5.00 ± 1.56a	1.53 ± 0.19cd	2.00 ± 0.41b	3.90 ± 0.20b	6.00 ± 0.61ab
	0.2	5.00 ± 1.56a	1.03 ± 0.19ef	2.00 ± 0.41b	3.00 ± 0.20e	4.33 ± 0.61def
	0.4	6.67 ± 1.56ab	1.03 ± 0.19ef	1.00 ± 0.41c	2.53 ± 0.20f	3.33 ± 0.61fgh

Means in the same column followed by the same letters are not significantly different ($P < 0.05$)

As shown by the height of shoots and number of leaves, subsequent growth of the plantlets was also strongly influenced by the growth regulators (BAP and NAA) in the culture medium. After 15 days of culture, the height of the shoots decreased as the concentration of both BAP and NAA in the culture medium increased (Table 5.1). The optimal concentration of the growth regulators for rapid shoot development was 2.0 mg/l BAP when NAA concentration was 0.1 mg/l (Fig. 5.6). At this concentration, height of the plantlets was significantly higher (2.60 cm) than those for all the remaining treatments where the height did not exceed 1.5 cm.

The number of leaves produced by the plantlets at 15 days of culture also decreased as the concentration of both growth regulators increased. Similar to shoot height, the optimal concentration for leaf production was 2.0 mg/l BAP and 0.1 mg/l NAA. This concentration of the growth regulators resulted in significantly higher number of leaves (3), albeit being the same as the number of leaves produced at BAP/NAA combination of 4.0 and 0.0 mg/l respectively (Table 5.1). At this concentration of BAP (4.0 mg/l), leaf production clearly decreased as NAA concentration increased resulting in the least number of leaves (1).

At 45 days of culture, height of plantlets almost doubled at the optimal concentration of 2.0 mg/l BAP and 0.1 mg/l NAA. For leaf production, a BAP/NAA combination of 2.0 and 0.2 mg/l respectively resulted in the highest number of leaves (7). However, no significant difference occurred between this highest number of leaves and that produced by a BAP and NAA combination of 4.0 mg/l and 0.1 mg/l respectively.

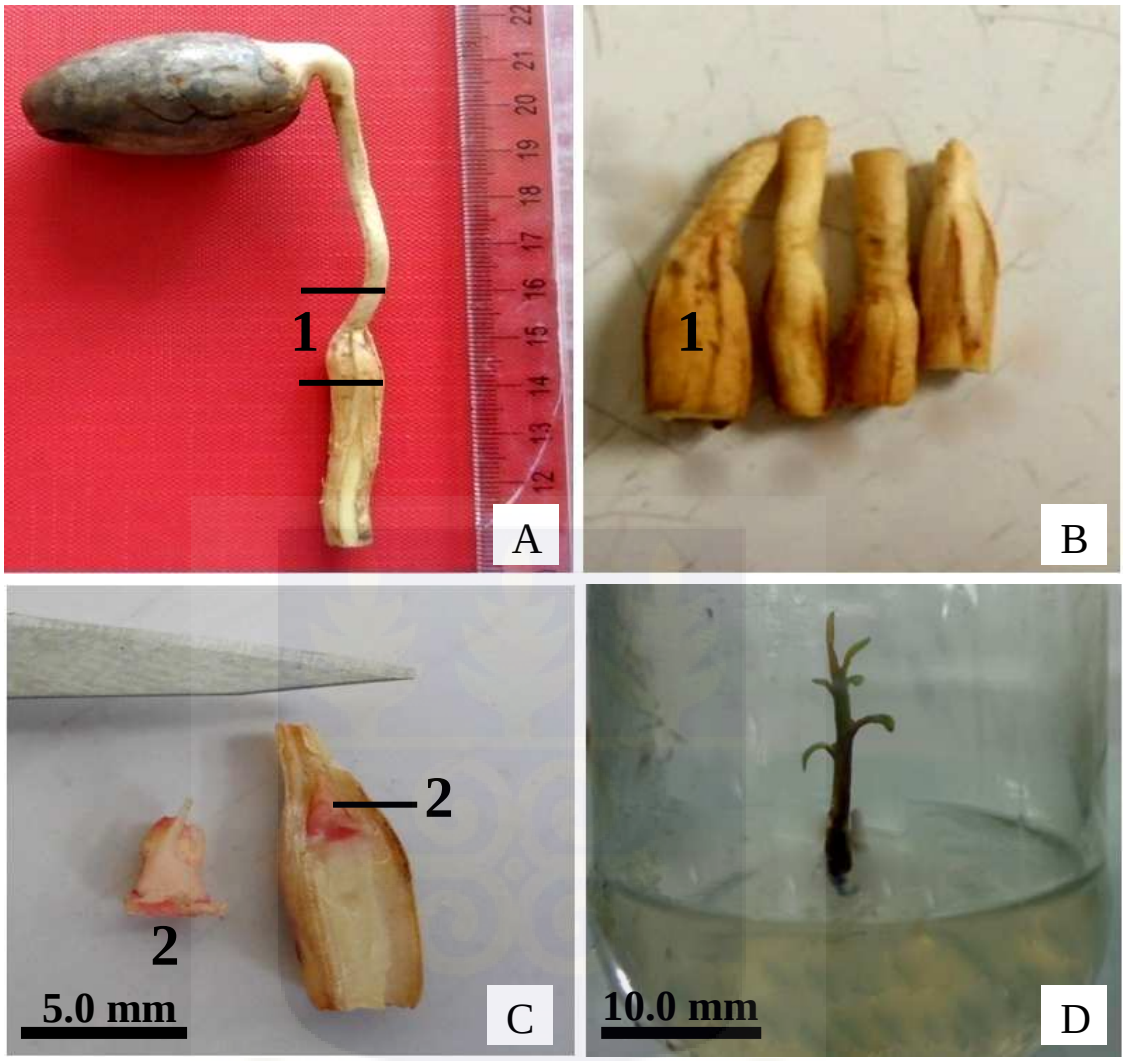

Fig. 5.6. *In vitro* regeneration of *V. paradoxa* using rudimentary shoots; A, Pseudoradicle showing the bulge (1); B, Excised bulges of the seedlings containing rudimentary shoots (1); C, Dissected bulge showing rudimentary shoot (2) used as explant; D, Regenerated plantlet from a rudimentary shoot 15 days after culture on MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l NAA

Morphologically, the plantlets produced showed distinct shoots with leaves similar to those produced by *in vivo* seedlings except that they had no roots. Some of the plantlets even showed signs of producing multiple shoots (Fig. 5.7A). In contrast to *in vivo* seedlings, rudimentary shoot explants produced scale leaves all of which expanded upon culture becoming green and fully functional leaves.

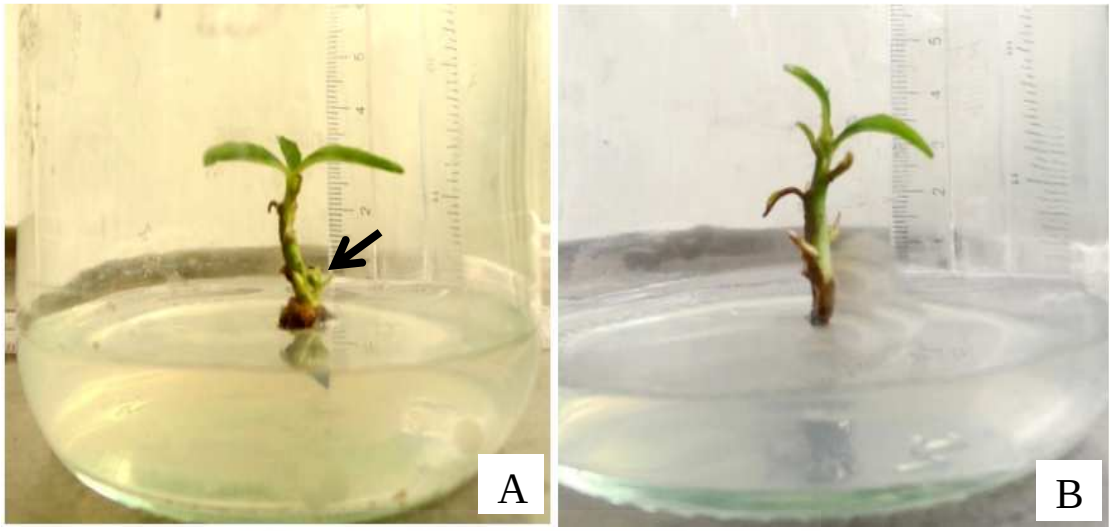

Fig. 5.7. Regenerated *Vitellaria paradoxa* shoots cultured on MS basal medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA at 30 days after culture; A, Shoot with a lateral shoot (arrowed) just beginning to develop; B, Shoot with expanded leaves

5.4. Discussion

In vitro regeneration of *V. paradoxa* will immensely accelerate the domestication of this economically important species and thereby improve the socioeconomic life of the majority of people especially women whose livelihood depends on it. However, to-date, little effort has been made to domesticate, or to establish plantations of shea because the tree grows in the wild and has largely been regarded as such (Gwali *et al*., 2012). *Vitellaria paradoxa* has the ability to grow abundantly on marginal soils and to improve soil quality due to annual leaf shedding (Dianda *et al*., 2009). With these potentials, *V. paradoxa* should be urgently considered for use for both afforestation and reafforestation programmes because the vegetative cover is being rapidly destroyed by bushfires. This forestation programme may, however, require the use of alternative modes of propagating this species. This study was thus aimed at developing efficient *in vitro* regeneration techniques for *V. paradoxa*.

Plantlets were never produced, albeit signs of sprouting and development of pseudoradicles, when intact and deshelled seeds were cultured respectively. For deshelled seeds, pseudoradicle development and elongation were significantly ($P < 0.05$) influenced by the presence of BAP in the culture medium because the days to sprouting were reduced from 2 weeks on high BAP (3.0 or 4.0 mg/l) amended medium to 1 week on low concentration (1.0 or 2.0 mg/l) of the growth regulator. Similarly, the percentage number of seeds that developed pseudoradicles was also significantly reduced as the BAP concentration increased. At higher concentrations, phytotoxicity of BAP on plant tissues has already been observed in plants such as *Gladiolus* (Shaheenuzzaman *et al*., 2011). Thus, the delay in sprouting and decrease in sprouting percentage as BAP concentration increased might also be due to the phytotoxic effects of the growth regulator on *V. paradoxa*.

Also, the failure of sprouted seeds to develop shoots may be attributed to the phenolic compounds they exuded, which eventually caused them to turn brown. Exudates of phenolic compounds cause excessive browning or necrosis and subsequent death of explants (Abdelwahd *et al*., 2008; Dibax *et al*., 2005). The phenolics are usually produced as metabolites during the growth of the plant (Arnaldos *et al*., 2001) and in the case of *V. paradoxa*, their production rate was probably high because some of the sprouted deshelled seeds turned brown as early as 28 days after culture despite pre-soaking in antioxidant solutions for 3 hours.

Another contributing factor to the poor development of shoot *in vitro* may be the presence of latex. Bhore and Preveena (2011) observed 100 % contamination of explants during *in vitro* propagation of *Mimusops elengi* (an Asian Sapotaceae)

because of the latex excreted by the explants. They concluded that the latex could hardly be taken out from the culture medium completely because the plant produced it during growth. Consequently, the contamination of the medium with white sap visible on the exposed parts of the pseudoradicles could have been caused by the latex produced by the explants as they were developing.

The culture of embryonic axes stained with TTC also resulted in a higher production of pseudoradicles than those observed in the deshelled seeds. The response of the embryonic axes to BAP in the culture medium was also different from those of the intact seeds. The optimal concentration of BAP that enhanced sprouting was 2.0 mg/l, but sprouting decreased to almost half (50 %) at the highest concentration of BAP (4.0 mg/l) again suggesting the phytotoxic effects of the growth regulator.

Yet again, full plantlets were never regenerated in embryonic axis culture although latex-related contamination was eliminated. In contrast, whole seedlings were successfully regenerated *in vitro* from zygotic embryos of *M. elengi* (Bhore and Preveena, 2011). One of the factors that most likely accounted for the inability to regenerate whole plantlets could be the cryptogeal morphology of *Vitellaria* seedlings. The cryptogeal morphology which indicates embryo development taking place in the pseudoradicle as germination progresses could place a genetic barrier on embryo development outside the pseudoradicle. Cryptogeal seedling development remains unreported for any other Sapotaceae (Jackson, 1974).

Although embryonic axes failed to produce full plantlets, rudimentary shoots excised from the pseudoradicles and cultured on the same MS medium developed plantlets

with well-distinct shoots and leaves. The development of the plantlets was influenced by the presence of BAP and NAA in the regeneration medium. The growth regulators had a significant effect ($P < 0.05$) on the response of rudimentary shoot explants to culture conditions and subsequent development of both shoots and leaves. Increasing the concentration of BAP produced a significantly faster response and subsequently resulted in faster growth in terms of both shoot height and number of leaves produced. Effects of NAA at lower concentrations (0.1 or 0.2 mg/l) were stimulating on both growth parameters, whilst the highest dose (0.4 mg/l) inhibited growth.

A BAP concentration of 2.0 mg/l was optimal giving significantly taller shoots and a higher number of leaves. A combination of 2.0 mg/l BAP with 0.1 or 0.2 mg/l NAA giving a cytokinin/auxin ratio ranging from 10:1 to 20:1 was the optimum concentration of the growth regulators for both shoot and leaf production. Lovett and Haq (2013) had earlier observed maximum shoot regeneration of *V. paradoxa* at a high BA/NAA ratio between 5:1 and 50:1. According to Kalidass and Mohan (2009), cytokinins have phytotoxic effects on shoot production and on growth of many plants, and this phytotoxicity may also be true for *V. paradoxa* because shoot height and leaf production were significantly reduced at higher BAP concentrations (3.0 or 4.0 mg/l).

On the medium with BAP/NAA combination of 2.0 mg/l and 0.2 mg/l respectively, the shoots grew up to 6.67 cm tall 45 days after culture. Asante *et al*. (2012) recorded a mean seedling height of 4.66 cm at 168 days after sowing suggesting that above-ground growth of *V. paradoxa* seedlings under *ex vitro* nursery conditions is slow. Thus, the production of plantlets as tall as 7.0 cm in just 45 days of culture may

indicate accelerated growth of the plants in culture. *In vitro* propagation thus has the potential to speed up regeneration of *V. paradoxa* for domestication.

Despite the rapid growth of shoots, the regenerated shoot plantlets produced no roots after 45 days of culture. Lovett and Haq (2013) also observed no root development when axillary shoot tips of *V. paradoxa* were cultured for 42 days on a medium that contains a high combination of BA/NAA. The failure of plantlets to produce roots as observed in this study might probably be due to the high cytokinin concentration in the medium, but this claim requires further investigation. *In vitro* regeneration of *S. dulcificum* by Ogunsola and Ilori (2008) also demonstrates the need for high auxin/cytokinin ratio for root production, at least in the Sapotaceae.

5.5. Conclusion

Rapid *in vitro* regeneration of *V. paradoxa* was achieved by using rudimentary shoots as explant. A BAP concentration of 2.0 mg/l in combination with 0.1 or 0.2 mg/l NAA was optimal for shoot and leaf production. Rudimentary shoots may, therefore, be ideal explants for *in vitro* regeneration of *V. paradoxa* via organogenesis and somatic embryogenesis. This protocol is both simple and cost-effective because it significantly minimizes latex-associated contamination. The rudimentary shoot explants also have the potential of producing multiple shoots and may thus enable superior genotypes to be rapidly multiplied on large-scale basis for farmers.

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CHAPTER 6

6.0. General conclusions and recommendations

6.1. Conclusions

Vitellaria paradoxa is an indigen of sub-Saharan Africa and became a plant of international trade in the early 1950s when the butter extracted from its kernel was discovered as one of the best cocoa butter substitutes. Much of the research work on *V. paradoxa* has been primarily focused on exploiting it. Despite its socioeconomic value of sustaining the livelihood of women and children, it still remains undomesticated largely due to the non-availability of reliable methods of producing planting materials commercially and its long gestation period.

This study sought to develop appropriate techniques for propagating *V. paradoxa* as part of initial efforts towards hastening its domestication. The following conclusions have been made from the study:

1. The embryo of *Vitellaria paradoxa* seed is located at the proximal end where it is embedded in copious amount of latex and fatty tissues making its identification and isolation difficult.
2. The seed is proximally syncotylous which accounts for the production of long and fused cotyledonary petioles (pseudoradicles) during germination.
3. *Vitellaria paradoxa* seedlings developed through 7 distinct stages namely: sprouting of the seed, elongation of the pseudoradicle, formation of a bulge in which the plumule develops into a rudimentary shoot, appearance of the rudimentary shoot on the pseudoradicle, elongation of the shoot, emergence and establishment.

4. Seed size had significant effect on seedling development because large seeds produced seedlings with shorter taproots and vigorous above-ground growth, whilst small and medium seeds produced seedlings with longer taproots.
5. Seedlings produced from deshelled seeds emerged faster and more synchronously than those produced by intact seeds.
6. Seedlings produced by the deshelled seeds developed large tuberous root crowns from which lateral shoots developed. These seedlings also produced several axillary shoots making them shrubby.
7. Amongst the 3 different types of explants cultured, only *in vivo* rudimentary shoots successfully and rapidly developed into plantlets.
8. Murashige and Skoog basal medium supplemented with 2.0 mg/l BAP and 0.1 or 0.2 mg/l NAA was the optimum concentration of the growth regulators for shoot regeneration because 86 % of the cultured rudimentary shoot explants developed into plantlets.

6.2. Recommendations

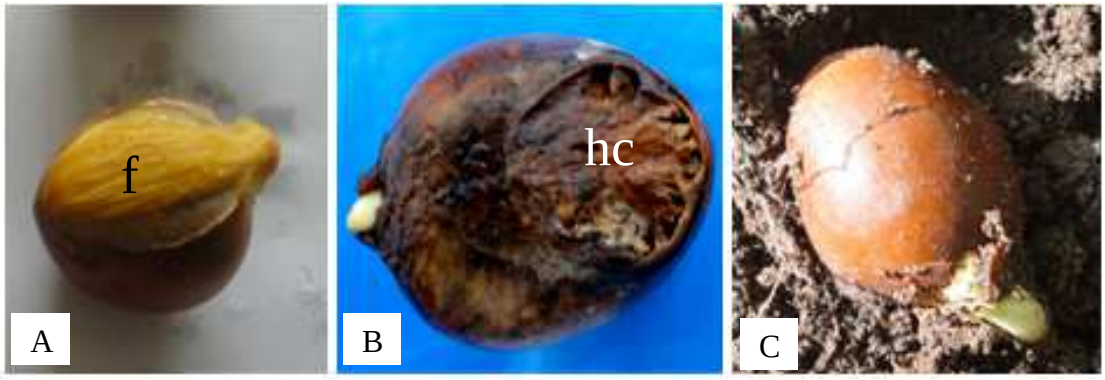
Based on the findings of this study, the following recommendations would be very useful for developing appropriate techniques for propagating *Vitellaria paradoxa*.

1. The effect of seed storage on viability should be investigated. This study and its findings will facilitate international germplasm exchange.
2. Further investigations are required to achieve rooting of regenerated rudimentary shoots *in vitro*.
3. Rudimentary shoots should be used as explants for somatic embryogenesis.
4. The induction of multiple shoots from rudimentary shoot explants should be further investigated.

APPENDICES

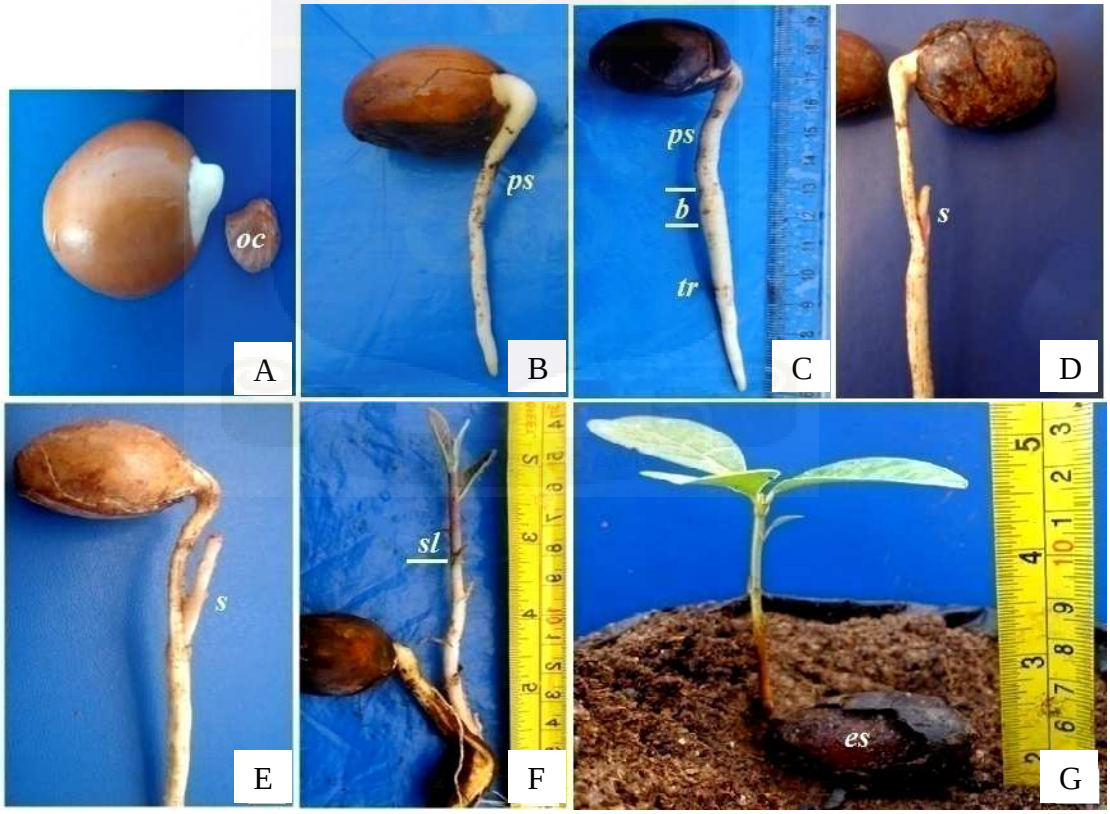
7.0. Appendices

Appendix 7.1A. Seeds of *Vitellaria paradoxa*



A, Seed showing the bitter-tasting funiculus (f); B, Seed showing the woody hilar cup (hc) eaten by xylophagus insects; C, Naturally dispersed seed lying hilum down

Appendix 7.1B. Stages of the development of *Vitellaria paradoxa* seedlings

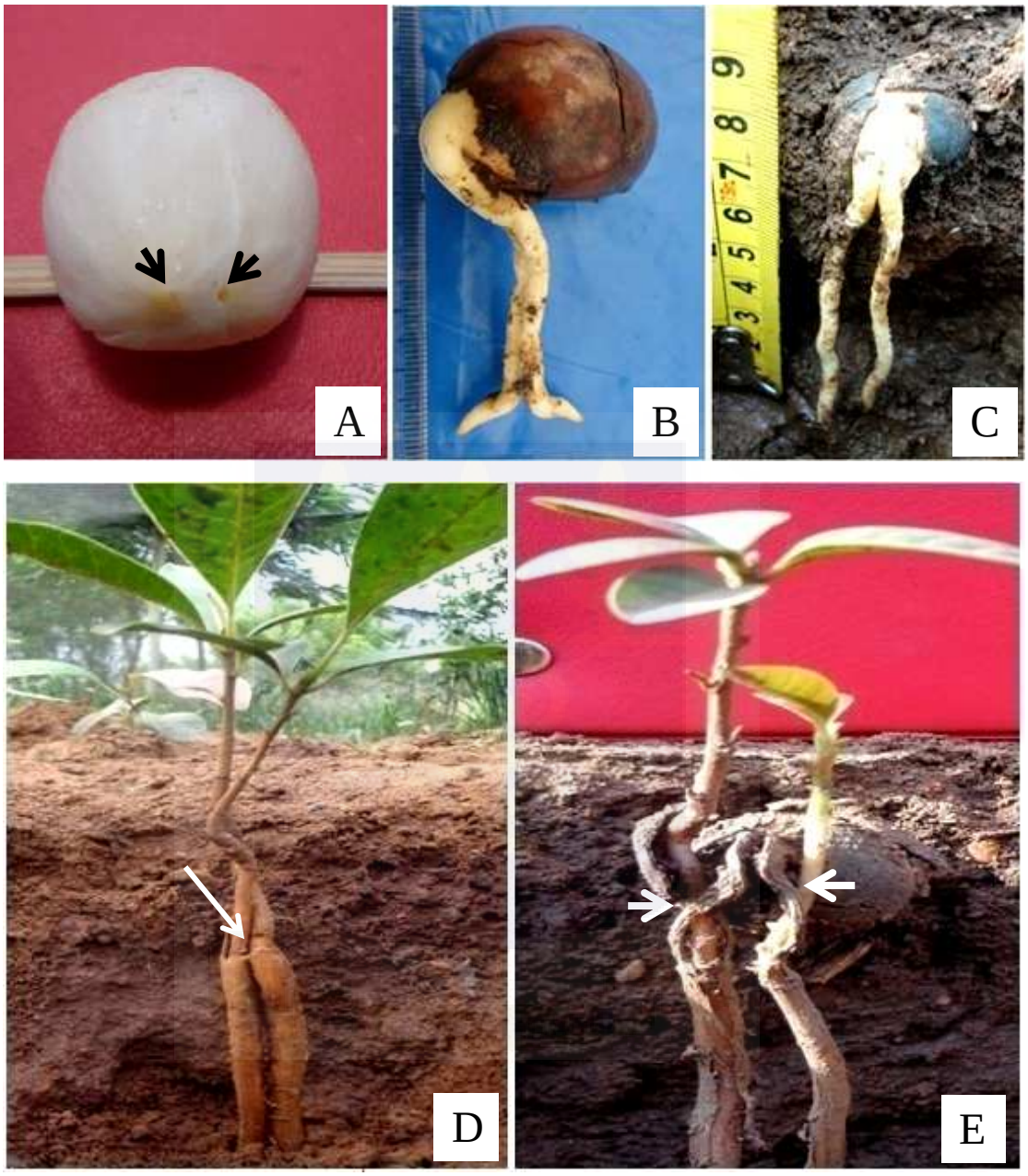


A, Sprouting; B, Pseudoradicle elongation; C, Bulging; D, Shoot appearance; E, Shoot elongation; F, Emergence; G, Establishment; Stages A–E are the skotomorphogenic growth stages; F and G are the photomorphorgenic growth stages; *oc*, operculum cap; *ps*, pseudoradicle; *b*, bulge; *tr*, true root; *s*, shoot; *sl*, soil level; *es*, exhausted seed

Appendix 7.2. Production of multiple shoots from monoembryonic seeds

A. Monoembryonic seed showing an unexserted embryo (arrowed) at the proximal end
B. Sprouted seed showing a pseudoradicle (arrowed)
C. Three shoots appearing from the cotyledonary slit
D. Three shoots that have emerged above the soil; cotyledonar raphe (arrowed)

Appendix 7.3. Production of multiple seedlings from polyembryonic seeds



A. Polyembryonic seed showing two embryos (arrowed)
B. Sprouted polyembryonic seed showing two appressed pseudoradicles
C. Sprouted polyembryonic seed showing two free pseudoradicles
D. Two seedlings conjoined to each other at the root–shoot junction (arrowed)
E. Two free seedlings still attached to the seed by their pseudoradicles (arrowed)

Appendix 7.4. ANOVA for effect of seed size on germination percentage

Source of variation	df	SS	MS	v.r.	F
Seed size	2	0.14222	0.07111	2.81	0.173
Replication	2	0.05056	0.02528	1.00	
Residual	4	0.10111	0.02528		
Total	8	0.29389			

Appendix 7.5. ANOVA for effect of seed size on emergence percentage

Source of variation	df	SS	MS	v.r.	F
Seed size	2	0.03909	0.01954	1.07	0.424
Replication	2	0.11209	0.05604	3.08	
Residual	4	0.07284	0.01821		
Total	8	0.22402			

Appendix 7.6. ANOVA for effect of seed size on emergence rate index

Source of variation	df	SS	MS	v.r.	F
Seed size	2	260.39	130.20	11.29	0.023
Replication	2	79.89	39.94	3.46	
Residual	4	46.14	11.54		
Total	8	386.42			

Appendix 7.7. ANOVA for effect of seed size on days to sprouting

Source of variation	df	SS	MS	v.r.	F
Seed size	2	30.9809	15.4904	23.95	0.006
Replication	2	3.1505	1.5752	2.44	
Residual	4	2.5867	0.6467		
Total	8	36.7180			

Appendix 7.8. ANOVA for effect of seed size on days to pseudoradicle elongation

Source of variation	df	SS	MS	v.r.	F
Seed size	2	149.959	74.980	8.94	0.033
Replication	2	9.534	4.767	0.57	
Residual	4	33.547	8.387		
Total	8	193.040			

Appendix 7.9. ANOVA for effect of seed size on days to formation of bulge on the pseudoradicle

Source of variation	df	SS	MS	v.r.	F
Seed size	2	79.5475	39.7737	48.15	0.002
Replication	2	1.8224	0.9112	1.10	
Residual	4	3.3040	0.8260		
Total	8	84.6739			

Appendix 7.10. ANOVA for effect of seed size on days to appearance of the shoot on the pseudoradicle

Source of variation	df	SS	MS	v.r.	F
Seed size	2	244.485	122.242	88.84	<.001
Replication	2	12.825	6.413	4.66	
Residual	4	5.504	1.376		
Total	8	262.814			

Appendix 7.11. ANOVA for effect of seed size on days to seedling emergence

Source of variation	df	SS	MS	v.r.	F
Seed size	2	318.891	159.445	17.02	0.011
Replication	2	9.174	4.587	0.49	
Residual	4	37.464	9.366		
Total	8	365.529			

Appendix 7.12. ANOVA for effect of seed size on days to seedling establishment

Source of variation	df	SS	MS	v.r.	F
Seed size	2	511.15	255.57	20.17	0.008
Replication	2	13.74	6.87	0.54	
Residual	4	50.68	12.67		
Total	8	575.57			

Appendix 7.13. ANOVA for effect of seed size on the length of the pseudoradicle at bulge formation

Source of variation	df	SS	MS	v.r.	F
Seed size	2	14.5204	7.2602	17.76	0.010
Replication	2	1.8074	0.9037	2.21	
Residual	4	1.6349	0.4087		
Total	8	17.9627			

Appendix 7.14. ANOVA for effect of seed size on the length of the taproot at bulge formation

Source of variation	df	SS	MS	v.r.	F
Seed size	2	23.9654	11.9827	13.02	0.018
Replication	2	1.6177	0.8088	0.88	
Residual	4	3.6816	0.9204		
Total	8	29.2647			

Appendix 7.15. ANOVA for effect of seed size on the length of the taproot at emergence

Source of variation	df	SS	MS	v.r.	F
Seed size	2	276.604	138.302	72.74	<.001
Replication	2	4.107	2.054	1.08	
Residual	4	7.605	1.901		
Total	8	288.316			

Appendix 7.16. ANOVA for effect of seed size on the total shoot height three weeks after emergence

Source of variation	df	SS	MS	v.r.	F
Seed size	2	3.8138	1.9069	7.20	0.047
Replication	2	1.3524	0.6762	2.55	
Residual	4	1.0590	0.2647		
Total	8	6.2252			

Appendix 7.17. ANOVA for effect of seed size on diameter of the root crown three weeks after emergence

Source of variation	df	SS	MS	v.r.	F
Seed size	2	0.1017556	0.0508778	194.85	<.001
Replication	2	0.0046889	0.0023444	8.98	
Residual	4	0.0010444	0.0002611		
Total	8	0.1074889			

Appendix 7.18. ANOVA for effect of seed size on diameter of the shoots 3 weeks after emergence

Source of variation	df	SS	MS	v.r.	F
Seed size	2	0.0150889	0.0075444	21.90	0.007
Replication	2	0.0021556	0.0010778	3.13	
Residual	4	0.0013778	0.0003444		
Total	8	0.0186222			

Appendix 7.19. ANOVA for effect of deshelling of the seeds on germination percentage

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	0.096	0.096	0.63	0.512
Replication	2	0.281	0.141	0.91	
Residual	2	0.308	0.154		
Total	5	0.685			

Appendix 7.20. ANOVA for effect of deshelling of the seeds on mean germination time

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	2.734	2.734	2.61	0.247
Replication	2	3.101	1.550	1.48	
Residual	2	2.092	1.046		
Total	5	7.927			

Appendix 7.21. ANOVA for effect of deshelling of the seed on emergence percentage

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	0.582	0.582	43.93	0.022
Replication	2	0.299	0.149	11.25	
Residual	2	0.027	0.013		
Total	5	0.908			

Appendix 7.22. ANOVA for effect of deshelling of seeds size on emergence index

Source of variation	df	SS	MS	v.r.	F
Deshelling of seeds	1	493.41	493.41	297.18	0.003
Replication	2	29.24	14.62	8.81	
Residual	2	3.32	1.66		
Total	5	525.97			

Appendix 7.23. ANOVA for effect of deshelling of the seeds on emergence rate index

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	1378.95	1378.95	92.95	0.011
Replication	2	185.07	92.53	6.24	
Residual	2	29.67	14.84		
Total	5	1593.70			

Appendix 7.24. ANOVA for effect of deshelling of seeds on mean stem diameter of seedlings 150 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	0.0054	0.00540	15.43	0.059
Replication	2	0.0019	0.00095	2.71	
Residual	2	0.0007	0.00035		
Total	5	0.0080			

Appendix 7.25. ANOVA for effect of deshelling of seeds on seedling height 150 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	0.84375	0.84375	1875.00	<.001
Replication	2	0.15790	0.07895	175.44	
Residual	2	0.00090	0.00045		
Total	5	1.00255			

Appendix 7.26. ANOVA for effect of deshelling of seeds on number of leaves produced by seedlings 150 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	1.7281	1.7281	3.81	0.190
Replication	2	0.0506	0.0253	0.06	
Residual	2	0.9076	0.4538		
Total	5	2.6863			

Appendix 7.27. ANOVA for effect of deshelling of seeds on mean leaf area of seedlings 150 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	288.57	288.57	3.44	0.205
Replication	2	231.16	115.58	1.38	
Residual	2	167.87	83.93		
Total	5	687.59			

Appendix 7.28. ANOVA for effect of deshelling of seeds on mean root crown diameter of seedlings 150 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	0.201667	0.201667	22.74	0.041
Replication	2	0.030400	0.015200	1.71	
Residual	2	0.017733	0.008867		
Total	5	0.249800			

Appendix 7.29. ANOVA for effect of deshelling of seeds on mean stem diameter of seedlings 240 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	0.49882	0.49882	49.55	0.020
Replication	2	0.12413	0.06207	6.17	
Residual	2	0.02013	0.01007		
Total	5	0.64308			

Appendix 7.30. ANOVA for effect of deshelling of seeds on mean height of seedlings 240 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	0.8588	0.8588	2.84	0.234
Replication	2	1.8097	0.9048	2.99	
Residual	2	0.6050	0.3025		
Total	5	3.2735			

Appendix 7.31. ANOVA for effect of deshelling of seeds on mean number of leaves produced by seedlings 240 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	4.034	4.034	0.93	0.437
Replication	2	6.546	3.273	0.75	
Residual	2	8.689	4.345		
Total	5	19.270			

Appendix 7.32. ANOVA for effect of deshelling of seeds on mean leaf area of seedlings 240 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	4763.5	4763.5	24.98	0.038
Replication	2	307.9	154.0	0.81	
Residual	2	381.3	190.7		
Total	5	5452.8			

Appendix 7.33. ANOVA for effect of deshelling of seeds on root crown diameter of seedlings 240 days after sowing

Source of variation	df	SS	MS	v.r.	F
Deshelling of seed	1	1.70667	1.70667	32.28	0.030
Replication	2	0.21280	0.10640	2.01	
Residual	2	0.10573	0.05287		
Total	5	2.02520			

Appendix 7.34. ANOVA for the effect of concentration of BAP in culture medium on duration to sprouting of deshelled seeds

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	212.500	70.833	7.56	<.001
Residual	44	412.500	9.375		
Total	47	625.000			

Appendix 7.35. ANOVA for the effect of concentration of BAP in culture medium on percentage number of sprouted deshelled seeds

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	12000	4000	1.69	0.176
Residual	76	180000	2368		
Total	79	192000			

Appendix 7.36. ANOVA for the effect of concentration of BAP in culture medium on duration to sprouting of embryonic axes

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	175.00	58.33	4.69	0.006
Residual	52	646.43	12.43		
Total	55	821.43			

Appendix 7.37. ANOVA for the effect of concentration of BAP in culture medium on GP embryonic axes

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	20000	6667	3.42	0.021
Residual	76	148000	1947		
Total	79	168000			

Appendix 7.38. ANOVA for factorial analysis of the effect of concentration of BAP and NAA in the culture medium on the duration to response of rudimentary shoots to culture conditions

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	239.062	79.688	21.86	<.001
Concentration of NAA	3	168.229	56.076	15.38	<.001
Conc. of BAP × Conc. of NAA	9	75.521	8.391	2.30	0.040
Residual	32	116.667	3.646		
Total	47	599.479			

Appendix 7.39. ANOVA for factorial analysis of the effect of concentration of BAP and NAA in the culture medium on height of regenerated rudimentary shoots 15 days after culture

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	2.27167	0.75722	15.87	<.001
Concentration of NAA	3	4.19833	1.39944	29.33	<.001
Conc. of BAP × Conc. of NAA	9	2.85333	0.31704	6.65	<.001
Residual	32	1.52667	0.04771		
Total	47	10.85000			

Appendix 7.40. ANOVA for factorial analysis of the effect of concentration of BAP and NAA in the culture medium on number of leaves produced by regenerated rudimentary shoots 15 days after culture

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	2.7292	0.9097	3.64	0.023
Concentration of NAA	3	6.7292	2.2431	8.97	<.001
Conc. of BAP × Conc. of NAA	9	5.8542	0.6505	2.60	0.022
Residual	32	8.0000	0.2500		
Total	47	23.3125			

Appendix 7.41. ANOVA for factorial analysis of the effect of concentration of BAP and NAA in the culture medium on height of regenerated rudimentary shoots 45 days after culture

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	20.74167	6.91389	119.38	<.001
Concentration of NAA	3	10.94167	3.64722	62.97	<.001
Conc. of BAP × Conc. of NAA	9	1.84000	0.20444	3.53	0.004
Residual	32	1.85333	0.05792		
Total	47	35.37667			

Appendix 7.42. ANOVA for factorial analysis of the effect of concentration of BAP and NAA in the culture medium on number of leaves produced by regenerated shoots 45 days after culture

Source of variation	df	SS	MS	v.r.	F
Concentration of BAP	3	29.4167	9.8056	17.43	<.001
Concentration of NAA	3	40.0833	13.3611	23.75	<.001
Conc. of BAP × Conc. of NAA	9	5.8542	1.3796	2.45	0.030
Residual	32	18.0000	0.5625		
Total	47	99.9167			