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Hormonal Profiling in Ungerminated and Germinated Seeds of African Yam Bean (*Sphenostylis stenocarpa* (Hochst. ex A. Rich.) Harms)

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ABSTRACT : Hormonal profiles of ungerminated and germinated seeds of *Sphenostylis stenocarpa* were studied. The hormone gibberellin (GA) was extracted with 80% methanol. The hormone was purified with polyvinyl pyrrolidone (PVP) column chromatography and quantified with high performance liquid chromatography (HPLC). High performance liquid chromatography analysis identified five endogenous gibberellins GA_1, GA_3, GA_4, GA_7 and GA_{20}. Quantification of gibberellin in ng/g (dw) for ungerminated and germinated seeds showed that GA_1 was highest in quantity than GA_3, GA_4, GA_7 and GA_{20} in all the fractions in ungerminated seed while GA_3 was highest in quantity in germinated seed than GA_1, GA_4, GA_7 and GA_{20}. This study suggests that hormone extracted from ungerminated and germinated seeds of *S. stenocarpa* could be a source of phytohormones for research and agricultural purposes.

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Gibberellins (GA_s) are known to accumulate in developing seeds from many species (Khan, 1982; Sponsel, 1983; Garcia –Martinez *et al,* 1987) and some studies have reported that exogenously applied GAs can induce precocious germination of immature embryos (Nortstog and Klein, 1972), their role in dertermining the germination capacity of developing seeds is by far less clear than in the case of ABA. Several authors have proposed that the dormancy level of a seed population depends on the balance between the action of promotive (GAs, Kinetins) and inhibitory (ABA) hormones (Khan, 1982). On the other hand, Karssen and Lacka (1986) concluded that the GAs do not play any significant role in the establishment of or exit from dormancy based on results with GA-deficient mutants. The same authors proposed that, in contrast to the hormone balance theory, GAs and ABA do not interact directly, that GAs are required for germination once dormancy is lost, whereas ABA levels influences the depth of dormancy during development.

Inhibition of GA biosynthesis during seed development mimics the effects of exogenous ABA, for example in suppressing vivipary. It appears to be the ABA: GA ratio, and not the absolute hormone contents, that control germination. Thus its seems that GA directly antagonizes ABA signaling during dormancy induction of cereal grains. While dormancy maintenance also depends on high ABA: GA ratios, dormancy release involves a net shift to increase GA biosynthesis and ABA degradation resulting in low ABA: GA ratios (Ali-Rachedi *et al.,* 2004); Cadman *et al.,* 2006). This supports the proposal of Le page – Degivry *et al* (1996) that ABA is the primary hormone involved in any step during dormancy maintenance and release, and that Gibberellins (GAs) are present at sufficient concentrations to promote germination as soon as ABA biosynthesis is inhibited. There is further support from genetic work with *Avena fatua* (Fennimore and Foley, 1998) showing that GA itself, although its addition to the medium can cause germination of dormant seeds, is not involved in embryo dormancy loss, but in stimulating seed germination. Thus, dormancy release is characterized by the capacity for enhanced ABA degradation and increased GA biosynthesis, which is followed by GA promotion of seed germination.

The African yam bean (*Sphenotylis stenocarpa* Hochst. ex. A. Rich) is a leguminous crop belonging to the family Fabaceae, sub-family papilonoidae, tribe Phaseoleae, sub-tribe Phaseolinae and genus *Sphenostylis* (Okigbo, 1973; Allen and Allen, 1987). It is grown as a minor crop in mixed association with yam and cassava. Its current low status as a minor crop means that this crop is largely unexploited (Klu *et al*, 2001). The African yam bean is grown in West Africa, particularly in Cameroon, Cote d'Ivoire, Ghana, Nigeria and Togo (Porter, 1992). In Nigeria, it is found in localized areas in the southern part of Nigeria, where it is grown by peasant farmers as a security

crop. It is in danger of extinction because of the high premium placed on the major legumes listed above and others such as Soya bean.

The Economic importance of African yam bean is immense. Increasing population, high prices of staple food items, policy constraints on food importation are worsening the food security in developing countries where protein deficiency and malnutrition is predominant (Weaver, 1994; FAO, 1994, 2008). In order to meet the increasing gap in the provision of balanced food for the growing population of developing countries, attention is now being paid to lesser –known crops that have played major roles in the livelihoods of subsistent rural farming families (Ezeagu *et. al*., 2002). Among these crops are African yam beans *Sphenostylis stenocarpa (*Hochst. ex A. Rich.) Harms and pigeon pea (*Cajanus cajan* L. Mill Sp.). They are grown for household consumption and for commercial purposes in Nigeria (Saka *et al*, 2004) despite their great potentials to meet adequate nutrition requirements. Such plants have variedly been referred to as under-exploited, under-utilized, orphan or neglected (Jaenicke *et al,* 2009). The nutritious seeds are delicious, and in most parts of Nigeria are often preferred over other leguminous seeds. In addition to its edible leaves and pods, the tubers can be used as cooked vegetable (Rice, *et al.,* 1986).

The hormonal profile of ungerminated and germinated seeds of *Sphenostylis stenocarpa* have not been clearly studied and documented. Therefore, the objective of this study is to investigate and outline the hormonal profile of ungerminated and germinated seeds of *Sphenostylis stenocarpa*.

MATERIALS AND METHODS

*Extraction of Gibberellin***:** The method of Esenowo (2004) was adopted. Ungerminated and germinated seedlings were pounded with a mortar to reduce seeds and seedlings into small pieces. One hundred grams (100 g) of powered seeds and seedlings was macerated with 500 ml of 80% methanol for 72 hours. The homogenate was extracted at 4^{o}C with continuous stirring. The combined extract 350ml was filtered under vacuum. The methanolic filtrate was thereafter reduced *in vacuo* to an aqueous volume (20 – 30 ml) in a rotatory evaporator at 35^{o}C. An equal volume of 0.5 molar phosphate buffers (pH 8.0) was added to the aqueous fraction and the combine volumes adjusted to pH 8.0 with 2M NaoH to ensure maximum solubility of gibberellins. The aqueous extract was partitioned 5 times against N-hexane (b.p 67.7 – 69.2) to remove lipids. The aqueous fraction was frozen for 12 hours, thawed and centrifuged at 4,000 r.p.m for 30 minutes at 4^{o}C to remove any sediments. The resulting supernatant was again adjusted to pH 8.0 for column chromatography.

Polyvinylpyrrolidone (P.V.P) Column Chromatography: The polymer, P.V.P (60 – 120 mesh) Sigma Chemical Company, U.S.A) was washed 5 times with acetone to remove any suspended fine particles. The slurry was packed in a glass column (60 x 3.0 cm) to height of 30 cm, and equilibrated with 0.1M phosphate buffer (potassium salt, pH 8.0). The extract was applied to the bed of the column and eluted with 500ml of the phosphate buffer. A 350 ml fraction was collected and adjusted to pH 8.0 and partitioned three times against ethyl acetate. The basic ethyl acetate fraction (200 ml) obtained was reduced to dryness in vacuo and the residue dissolved in an aliquot of 35% ethanol for thin layer chromatography (Esenowo, 2004). The aqueous fraction obtained was adjusted to pH 2.5 and partitioned against ethyl acetate. The resulting acidic ethyl acetate fraction (200 ml) was partitioned with distilled water and later reduced *in vacuo* to about one quarter of its original volume. An excess of toluene ethyl acetate/water-ternary azeotrope. This was then reduced to dryness and the residue dissolved in an aliquot of 35% ethanol for subsequent thin layer chromatography (Esenowo, 2004).

The aqueous fraction from the last partition was adjusted to pH 2.5 and partitioned 3 times against n-butanol fraction (200 ml). This was reduced to dryness and the residue was dissolved in an aliquot of 35% ethanol for TLC, and HPLC

Thin Layer Chromatography for Gibberellin: Thin layer glass plates (20 x 20 cm) of 0.5 mm thick ness were prepared by using silica gel (Kiesegel 60G) and allowed to dry at room temperature. The plates were activated at 80^{o}C for 30 minutes. 5ml gibberellin containing samples were loaded on the plate with a Pasteur pipette, equilibrated overnight and then developed in an ascending manner in methanol: hexane (50:50 v/v) to a distance of 10cm from the origin. The plates were allowed to dry, heated at 80^{o}C for 10 minutes and the resulting bands were made visible under UV light at 254 nm (yellow bands). The bands made visible under UV light were marked and then scrapped from the plates into small test tube and then eluted with 3ml distilled water for 12 hours at 4^{o}C. The eluate was carefully pipetted into 5cm Petri-dishes containing Whatman No I filter paper disc and assayed for giberellin using the soybean hypocotyl bioassay technique (Bensen *et al.,* 1990).

Soybean Hypocotyl Bioassay for Gibberellin: The method of Bensen *et al.,* (1990) was used with modification. The growth of the soybean hypocotyl

has been shown to be directly proportional to the logarithm of the concentration of gibberllin-like substances. Soybean seeds (*Glycine max [*L.] Merr.) were sown in 5cm Petri dishes containing Whatman No.1 filter paper disc moistened with 2ml of distilled water. The seeds were allowed to germinate in light at 25ºC for 24 – 36 hours. Seedlings with radicle (2-4mm) were selected. Ten of these were placed on a single filter paper disc in 5cm Petri dishes containing the eluates from the chromatograms. These dishes were placed below a light source from two 1000Watt bulbs in a cupboard. Five control dishes contained all other materials except the hormone sample. The seedlings were removed after 72 hours. The initiating and final lengths of hypocotyls were recorded and the means of three replicate were calculated.

Separation and Quantification of the Hormone Gibberellin by High Performance Liquid Chromatography (HPLC): The modified methods of Stinemetz and Roberts (1984) were used. The extracted sample was reconstituted with 5% methanol in 0.20M acetic acid. It was centrifuged at 1200g for 12 minutes and later filtered twice through fluoropore filter. The aliquot was injected into the column attached to the waters 600E Multisolvent Delivery system. The extract was eluted in a linear methanol gradient (0 – 100% in 30 minutes) in 0.20M acetic acid at a temperature of 30ºC.

RESULTS AND DISCUSSION

Soybean Seedlings Hypocotyl Elongation Bioassay for Gibberellin (GA): Soybean seedlings (72-hr-old) grown in Petri dishes responded to gibberellin-like substances through promotion of elongation of hypocotyl. As the concentration of the hormone increased, there was increase in the length of hypocotyl (Figure 1).

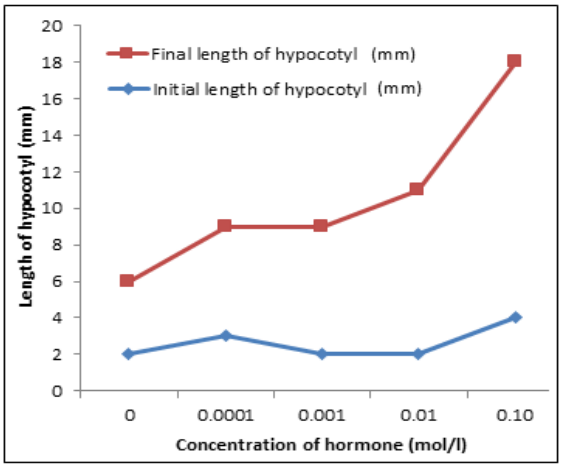

Fig 1: Hypocotyl elongation of soybean seedlings following treatment with GA

The events of dormancy and germination are controlled by a balance between hormones (Tipirdamaz and Gomurgen, 2000). Seeds of the same or different species may contain different levels of gibberellins, cytokinins and inhibitors leading to various severity of dormancy, from no apparent dormancy to absolute dormancy. Seed dormancy and germination are complex physiological processes that are controlled by a range of developmental and external cues (Ogawa, *et al.,* 2003). For germination to occur in physiologically dormant seeds, the mechanical constraint of the embryo-covering layers must be overcome by the growth potential of the embryo or the mechanical constraint must be reduced or both. A direct correlation between dormancy and a high endogenous Abscisic acid (ABA) content in tree seeds has been reported (Pinfield *et al.,* 1989; Chien *et al.,* 1998, 2004; Feurtada *et al.,* 2004; Chen *et al.,* 2007). Although the inhibiting effect of ABA on the seeds was apparent, the occurrence of and germination were not entirely the result of a reduction in the amount of endogenous ABA. However, gibberellin (GA_1 and GA_4) are highly active in promoting seed germination. Significant amounts of biologically active gibberellin-like activity were present in extracts from etiolated soybean hypocotyls. Soybean seedings (72-hr-old) grown in Petri dishes responded to gibberellin-like substances and showed elongation of hypocotyls over controls.

Quantification of Hormone Fraction by High Performance Liquid Chromatography (HPLC): Hormonal composition of ungerminated and germinated *S. stenocarpa* seed as determined by High Performance Liquid chromatography are presented in Tables 1 and 2 respectively. The results of the estimation of the gibberellin fractions as presented in tables 1 and were computed from HPLC analysis.

Identification and quantification of the gibberellin by high performance liquid chromatography (HPLC) allowed the major bioactive gibberellins (GAs) to be detected and characterized. In this study, reproducible patterns of GA-like bioactivity occurred after HPLC performed under one set of conditions. Compounds from these bioactive fractions coeluted in a linear methanol gradient gave the proper relative abundances for five major fragments. The fact that all of the gibberellins (GAs) recognized occurred in the same early 13-hydroxylation pathyway (Phinney, 1984) adds to the reliability of the work. Work with maize (Phinney, 1984; Spray *et al.,* 1984), pea (Ingram *et al.,* 1986), rice (Phinney, 1984) and Brassica (Rood *et al.*, 1989) mutants implicate the early 13 –hydroxylation pathway, particularly gibberellin, in the regulation of shoot elongation (Phinney, 1984).

Table 1: Hormonal composition of ungerminated seed of *S. stenocarpa*

Hormone fraction	GA_1	GA_3	GA_4	GA_7	GA_{20}
Basic ethyl acetate fraction	264.05006	71.39626	34.03488	46.92831	63.04325
Acidic ethyl acetate fraction	290.80095	73.55764	35.49708	58.31677	67.28511
n-butanol fraction	322.87225	78.09436	37.22235	64.07604	84.19039
Petroleum ether fraction	-	-	-	-	-
N-hexane fraction	-	-	-	-	-

Data shown have been computed from Figs. 5-7

Table 2: Hormonal composition of germinated seed of *S. stenocarpa*

Hormone fraction	GA_1	GA_3	GA_4	GA_7	GA_{20}
Basic ethyl acetate fraction	173.70056	502.18991	37.22235	3.20012	1.79474
Acidic ethyl acetate fraction	191.86927	572.45244	39.89684	4.08000	4.05621
n-butanol fraction	203.12120	665.69507	42.41081	6.24863	3.75317
Petroleum ether fraction	-	-	-	-	-
N-hexane fraction	-	-	-	-	-

Data shown have been computed from Figs. 2-4

Key: *GA_1= Gibberellin$_1$; GA_3= Gibberellin$_3$; GA_4= Gibberellin$_4$; GA_7= Gibberellin$_7$; GA_{20}= Gibberellin$_{20}$*

Conclusion: The use of high performance liquid chromatography (HPLC) for quantitative measurement of endogenous plant hormones in crude plant extracts, provide high sentivity, specificity, accuracy, and reproducibility. This study has established some protocols for extraction, identification, characterization and quantitative analyses of major plant hormone Gibberellin (GA) from crude plant extracts of *S. stenocarpa*.

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