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Extraction and Quantification of Abscisic Acid from Ungerminated and Germinated Seeds of Sphenostylis stenocarpa (Hoechst. ex A. Rich.) Harms.

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ABSTRACT

Extraction and quantification of Abscisic acid was carried out from ungerminated and germinated seeds of Sphenostylis stenocarpa. Abscisic acid (ABA) 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 and quantified the Abscisic acid. The result shows that ungerminated seeds contain higher quantity of ABA than germinated seeds which indicate that there was a drop in the level of ABA in germinated seeds. This study suggests that abscisic acid extracted from ungerminated and germinated seeds of S. stenocarpa could be a source of phytohormones for research and agricultural purposes.

Keywords: Extraction, Quantification, Abscisic acid, Sphenostylis stenocarpa.

INTRODUCTION

There is considerable evidence that Abscisic acid (ABA) is an important positive regulator of both the induction of dormancy and the maintenance of the dormant state in imbibed seeds following shedding. This evidence of ABA involvement has been described in detail by Kucera et al [1] and in several earlier reviews [2-5]. In most seeds the ABA level increases steadily during development reaching high values before ripening and then declining sharply [6] ABA deficiency during seed development is associated with absence of primary dormancy in the mature seed whereas over expression of ABA biosynthesis genes can increase seed ABA contents and enhance seed dormancy or delay germination [7]. ABA produced by the seed itself during seed development can impose a lasting dormancy whereas maternal ABA or ABA application during seed development fails to induce lasting seed dormancy, but has other function [1,8] suggests that ABA synthesis in the embryo and in the endosperm both contribute to the induction of seed dormancy. High ABA contents are present in the imbibed seeds of the strongly dormants *A. thaliana* ecotype Cope Verde Island (Cvi) and decrease as dormancy is lost [9]. A recent transcriptome analysis with this ecotype strongly supports the view that increased ABA biosynthesis is associated with the dormant state [10].

The addition of physiological concentration of ABA prevents precocious germination blocks the expression of germination-specific enzymes and promotes embryonic development [6,11-14] De novo ABA biosynthesis during imbibition of dormant and non dormant, seeds has been demonstrated in the *A. thaliana* ecotype cvi [9] as well as in other species including *Nicotiana plumbaginifolia* [15] *Helianthus annus* [16] and *Hordeum vulgare*. This de novo ABA biosynthesis was interpreted as a mechanism for dormancy maintenance.

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 [17,18]. 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 [19]. The African yam bean is grown in West Africa particularly in Cameroon Cote d'Ivoire, Ghana, Nigeria and Togo [20] 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[21-23]. 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 [24]. 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 [25] despite their great potentials to meet adequate nutrition requirements. Such plants have variedly been referred to as under-exploited under-utilized orphan or neglected [26]. 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 [27]. Currently there is no detailed research activity on abscisic acid and it implication in seed dormancy of *S.stenocarpa*. This work aims to study such.

MATERIALS AND METHODS

Extraction of abscisic acid

**********Ungerminated and germinated seeds were pounded with a mortar to reduce seeds into small pieces. One hundred grams (100 g) of powdered seeds was macerated with 500 ml of 80% methanol for 72 hours. The homogenate was extracted at 40℃ with continuous stirring. The combined extract 350 ml was filtered under vacuum. The methanolic filtrate (450 ml) obtained was reduced in vacuo to an aqueous fraction. It was adjusted to pH 8.0 with 2 M NaOH. This pH adjustment was to mimimize the isomerization of the cis-to trans ABA and to ensure ionization thereby preventing any loss by co-precipitation with water insoluble materials. This aqueous fraction (pH 8.0) was frozen for 12 hours thawed and centrifuged at 4000 rpm for 30 minutes at 4℃ to remove any sediments.The supernatant (20-40 ml) was adjusted to pH 3.5 and partitioned 3 times against equal volume of hexane. The combined hexane fraction was partitioned against 5% sodium bicarbonate solution to separate ABA from non-acidic substances. The bicarbonate fraction was then adjusted to pH 3.5 and partitioned 3 times against hexane. The combined hexane fraction was then dried overnight over anhydrous sodium sulphate and thereafter evaporated in vacuo to a small volume (3-5 ml) for PVP column chromatography.

Polyvinylpyrrolidone (PVP) column chromatography

The PVP slurry was prepared as for gibberellins. It was packed under gravity in a column (60 x 3.0 cm) to a height of 30 cm. Equilibration of the packed column was in 0.1 M phosphate buffer (pH 6.0). An equal volume of 0.1 M K_2HPO_4 solution was added to the extract before it was carefully applied to the bed of the column. Elution was with the same buffer the eluate (350 ml) collected was adjusted to pH 3.5 and partitioned 3 times against petroleum ether. The ether fraction was dried overnight over anhydrous sodium sulphate and thereafter reduced to dryness. The residue was then dissolved in an aliquot of 35% ethanol for TLC bioassay and HPLC.

Thin layer chromatography for abscisic acid

Thin layer plates were prepared from a slurry of silica gel in distilled water (silica gel: water 1:2). The slurry was spread to a thickness of 0.5 mm on glass plates 20 x 20 cm allowed to set and activated at 100℃ for 30 minutes. Samples containing abscisic acid (5 ml) were loaded with Pasteur pipette equilibrated overnight and developed in an ascending manner in methanol: hexane (50 : 50 v/v) to a distance of 15 cm. The plates were allowed to be barely dry and the resulting bands were made visible under UV light at 254 nm (purple bands). The bands made visible under UV light were marked and then scrapped from the plates into small Petri-dishes (5 cm in diameter) containing 3 ml distilled water. Elution was allowed for 12 hours at 4℃. The eluate was pipetted into 11 cm Petri-dishes containing Whatman No. 1 filter paper disc for assay using the cucumber cotyledons bioassay technique [28].

Cucumber greening cotyledons bioassay for abscisic acid

The method of Fletcher et al. [28] was used for Cucumber (*Cucumus sativus L*.) seeds were planted in small polythene bags filled with loamy soil and germinated in the dark at 28℃ for 5 days. At this point the seedlings were approximately 7 cm high, and the cotyldedons were excised in dim green light, making certain that the hypocotyl hook was removed. Groups of 15 cotyledons were placed in 5 cm Petri-dishes containing the eluates from the chromatograms. The Petri-dishes with the cotyledons were incubated in the dark at 28°C for 20 hours. They were then exposed to two 1000 Watt bulbs in a cupboard for 5 and 24 hours for chlorophyll determinations. The cotyledons were homogenized in 10 mL 80% acetone and then centrifuged at 2500 g for 10 minutes. The volume of the supernatant was brought up to 15 mL with acetone and the chlorophyll levels (on cotyledons basis) determined by measuring their absorbance at 663 nm. For fresh weight determinations the cotyledons were exposed to light for a period extending up to 72 hours [28].

Separation and quantification of abscisic acid by High Performance Liquid Chromatography (HPLC)

The modified methods of Stinemetz and Roberts were used [29] the extracted sample was reconstituted with 5% methanol in 0.20 M acetic acid. It was centrifuged at 1200 g for 12 minutes and later filtered twice through fluoropore

filter. The aliquot was injected into the column attached to the waters 600 E Multisolvent Delivery system. The extract was eluted in a linear methanol gradient (0 – 100% in 30 minutes) in 0.20 M acetic acid at a temperature of 30°C.

RESULTS

Cucumber cotyledons greening bioassay for Abscisic Acid (ABA)

The fresh weight of cucumber cotyledons incubated in the dark for 20 hours in the test solution at different concentrations and then exposed to light are shown in Table 1.

Table 1: Fresh weight of Cucumber Cotyledons incubated in the dark for 20 hrs (initial weight) in the test solution at different concentrations and then exposed to light

ABA	Light (h) ; Mg/cotyledon			
Concentration (Mol/L)	0	24	48	72
Control (butter pH5.8)	16	20	23	25
10 – 4	16	18	21	24
10 – 3	16	22	27	33
10 – 2	16	23	26	29
10 – 1	16	26	31	37

The results show that as the concentration of the abscisic acid-like substances increased, there was increase in the fresh weight of the cucumber cotyledons. Pretreatment of 5 day old etiolated cucumber cotyledons for 20 hours with abscisic acid in the dark and exposure to light for 5 hours inhibited chlorophyll production at all concentrations tested (Figure 1).

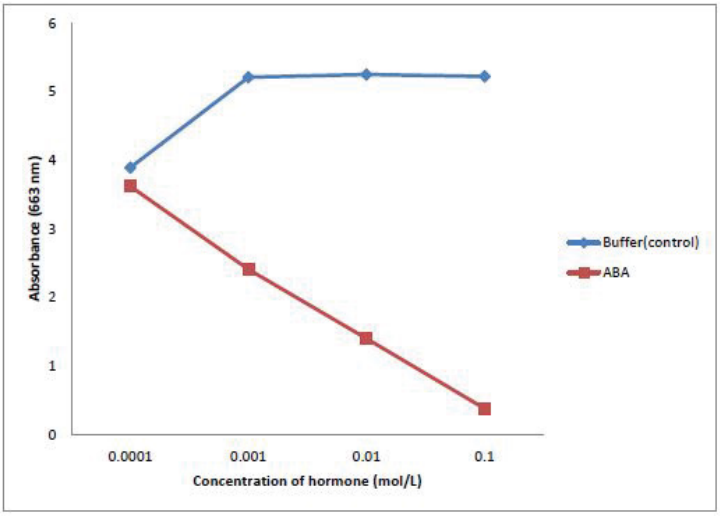


Figure 1: Chlorophyll levels in cucumber cotyledons treated with various concentrations of ABA.

Analytical Quantification of ABA Fraction by High Performance Liquid Chromatography (HPLC)

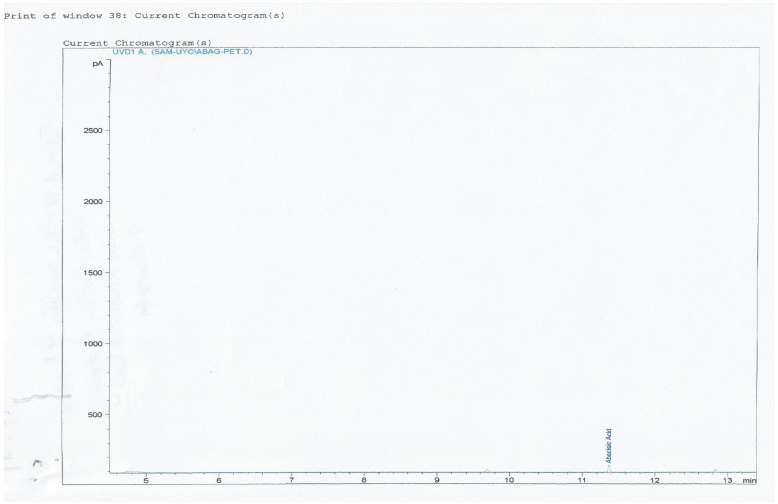
ABA composition of ungerminated and germinated *S.stenocarpa* seed as determined by High Performance Liquid chromatography (HPLC) is presented in (Table 2).

Table 2: ABA composition of germinated and ungerminated seed of *S. stenocarpa.*

ABA: Abscisic acid

Hormone fraction	ABA Germinated	ABA Ungerminated
Basic ethyl acetate fraction	_	_
Acidic ethyl acetate fraction	_	_
n-butanol fraction	_	_
Petroleum ether fraction	563.7	1650.0
N-hexane fraction	544.2	1743.1

Identification and quantification of absisic acid fractions are shown in (Figures 2 to 5). Data shown have been computed from (Figures 2 to 5).

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Dilution              :      1.0000

Signal 1: UVD1 A,

RetTime  Type      Area      Amt/Area     Amount    Grp    Name
 [min]           [pA*s]                [ng/g(dw)]
-------|------|----------|----------|----------|--|--------------------
 11.370 BP       92.40382     6.10143  563.79548  1 Abscisic Acid

Totals :                                563.79548

 Results obtained with enhanced integrator!
Group summary :

 Group  Use    Area       Amount       Group Name
  ID          [pA*s]   [ng/g(dw)]
|------|---|----------|----------|--------------------------|
  1          92.40382  563.79548  Abscisic Acid

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                          *** End of Report ***
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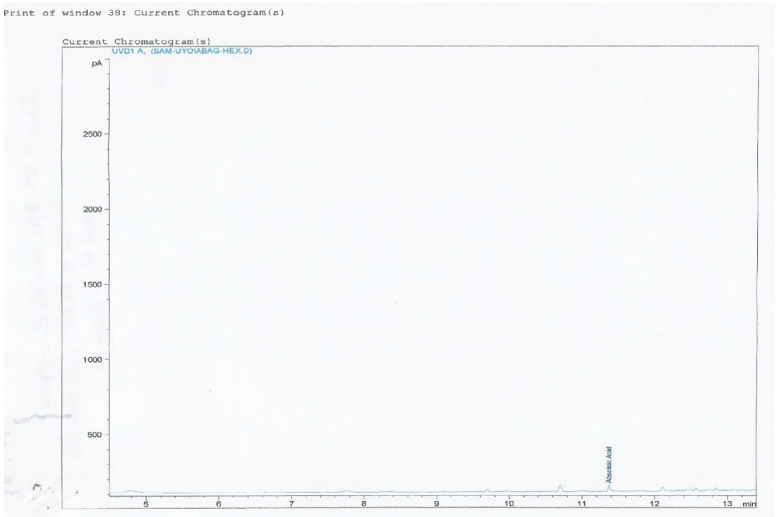
Figure 2: HPLC analysis of petroleum ether fraction of abscisic acid in germinated seed of *S. stenocarpa.*

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Signal 1: UVD1 A,

RetTime  Type      Area      Amt/Area     Amount    Grp    Name
 [min]           [pA*s]                [ng/g(dw)]
-------|------|----------|----------|----------|--|--------------------
 11.374 VV       123.71981     4.39893  544.23481  1 Abscisic Acid

Totals :                                544.23481

 Results obtained with enhanced integrator!
Group summary :

 Group  Use    Area       Amount       Group Name
  ID          [pA*s]   [ng/g(dw)]
|------|---|----------|----------|--------------------------|
  1          123.71981  544.23481  Abscisic Acid

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                          *** End of Report ***
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Figure 3: HPLC analysis of N-Hexane fraction of abscisic acid in germinated seed of *S.stenocarpa.*


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Dilution              :      1.0000

Signal 1: UVD1 A,

RetTime  Type     Area      Amt/Area    Amount   Grp   Name
  [min]         [pA*s]                [ng/g(dw)]
-------|------|----------|----------|----------|--|------------------
 11.369 BP       98.65925   16.72465 1650.04095  1 Abscisic Acid

Totals :                              1650.04095

 Results obtained with enhanced integrator!
Group summary :

 Group  Use     Area      Amount       Group Name
   ID          [pA*s]   [ng/g(dw)]
|------|---|----------|----------|--------------------------|
   1          98.65925 1650.04095  Abscisic Acid

================================================================================
                          *** End of Report ***
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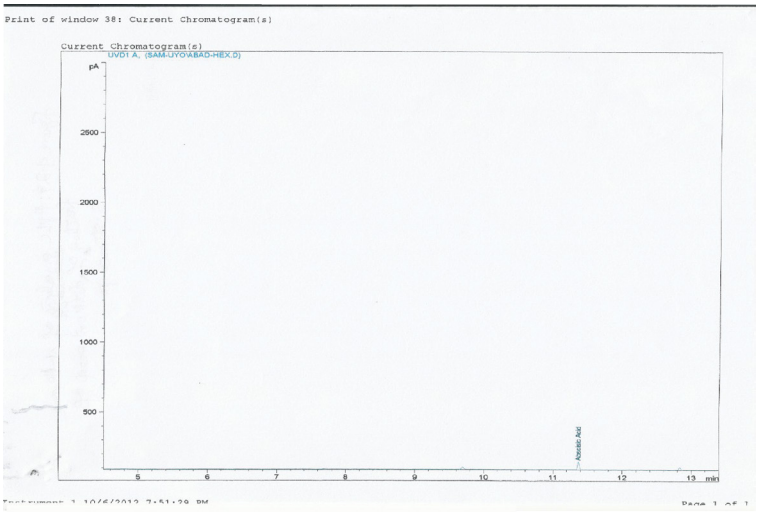
Figure 4: HPLC analysis of Petroleum ether fraction of abscisic acid in ungerminated seed of *S.stenocarpa.*

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Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: UVD1 A,

RetTime  Type     Area      Amt/Area    Amount   Grp   Name
  [min]         [pA*s]                [ng/g(dw)]
-------|------|----------|----------|----------|--|------------------
 11.374 VV       189.53209    9.19726 1743.17645  1 Abscisic Acid

Totals :                              1743.17645

 Results obtained with enhanced integrator!
Group summary :

 Group  Use     Area      Amount       Group Name
   ID          [pA*s]   [ng/g(dw)]
|------|---|----------|----------|--------------------------|
   1          189.53209 1743.17645  Abscisic Acid

================================================================================
                          *** End of Report ***
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Figure 5: HPLC analysis of N- Hexane fraction of abscisic acid in ungerminated seed of *S.stenocarpa.*

DISCUSSION

A direct correlation between dormancy and a high endogenous Abscisic acid (ABA) content in tree seeds has been reported [30-34]. 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. Inhibition of cotyledon growth by abscisic acid (ABA) has been reported by Longo et al [35]. It has been proposed that ABA interferes with potassium uptake [36]. Pretreatment of 5 day old etiolated cucumber cotyledons for 20 hours with absisic acid (ABA) in the dark and exposure to light for 5 hours inhibited chlorophyll production at all concentrations. Similar conclusions have been drawn by Ueda and Kato [37] who found that ABA inhibited the growth of radish cotyledons. Inhibition of chlorophyll production in pumpkin cotyledons by ABA has been reported previously [38] with the effect being more pronounced after longer exposure to light up to 5 days. In this study for chlorophyll determinations the cotyledons were not exposed to light for more than one day. After a lag phase of 3 hours the control cotyledons accumulated chlorophyll in a linear manner. This lag phase of 2 to 3 hours for etiolated tissue to produce chlorophyll after exposure to light is normal [39]. During this period the chloroplast are differentiating and synthesizing the necessary enzymes for chlorophyll synthesis and photosynthesis [40]. On the other hand ABA extends the lag phase to 6 hours and the amount of chlorophyll produced after 24 hours is 52% less than the control. Abscisic acid which is a potent inhibitor disrupts several processes associated with chlorophyll synthesis and destruction. In several systems ABA has been reported to antagonize cytokinin induced growth responses [41]. The decrease in chlorophyll level was found to be proportional to the concentration of ABA. Exposure of cotyledons to light for periods longer than 24 hours further increased the sensitivity of ABA enabling detections of concentrations lower than 10–4 M. Analytical quantification using 600 E multisolvent delivery system column was found suitable for ABA quantification in *S. stenocarpa* seed extract. The ABA quantification by the technique shows that the peaks from the analytical HPLC columns were infact only ABA. High performance liquid chromatography (HPLC) system has the ability to use large injection volumes, thus making it possible to quantify a larger portion of the total sample per injection. Quantification by analytical HPLC requires 40 times less sample dilution than by GLC-EC. However, one important note of caution is that the use of HPLC for identification and quantification of plant hormones may be quite tenuous unless the peaks being identified are first carefully characterized as being pure by either bioassay or preferably GC-MS [42].

CONCLUSION

Inhibition of chlorophyll production by abscisic acid-like substance is valuable as a simple and rapid bioassay for abscisic acid. The cucumber cotyledon greening system is ideal for detecting ABA which inhibits chlorophyll production. 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 ABA from crude plant extracts of *S. stenocarp.*

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