



## Extraction and Quantification of Cytokinin from Ungerminated and Germinated Seeds of *Sphenostylis stenocarpa* (HOCHST. EX A. RICH.) Harms

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**ABSTRACT:** Extraction and quantification of Cytokinin was carried out from ungerminated and germinated seeds of *Sphenostylis stenocarpa*. Cytokinin was extracted with methanol-chloroform-formic acid-water. The hormones were purified with polyvinyl pyrrolidone (PVP) column chromatography and quantified with high performance liquid chromatography (HPLC). High performance liquid chromatography analysis identified and quantified four endogenous cytokinins zeatins, trans-zeatin, isopentenyladenine, isopentenyladenosine. The ungerminated seeds of *S. stenocarpa* contain higher amount of zeatin, trans-zeatin, isopentenyladenine and isopentenyladenosine as compared with germinated seeds in all the hormone fractions. This study suggests that Cytokinin extracted from ungerminated and germinated seeds of *S. stenocarpa* could be a source of phytohormones for research and agricultural purposes.

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Cytokinins are a class of plant hormones promoting cell division and tissue differentiation (Rijavec *et al.*, 2009; Zhang, *et al.*, 2010). These endogenous compounds in plant tissues are considered as both local and long distance regulatory signals for the coordination of plant development (Kudo *et al.*, 2010), and are related to priming on water use efficiency and photosynthetic rate under conditions of salt stress (Iqbal and Ashraf, 2005). The motive of cytokinins are N<sup>6</sup>-substituted purine derivatives. Depending on the N<sup>6</sup>-substituent, these compounds can be classified into isoprenoid, modified isoprenoid and aromatic cytokinins. Cytokinins occur as nucleotides, nucleosides, bases and as conjugates with sugars at the 3-, 7-, or 9- position of the purine ring or at the N<sup>6</sup>-isoprenoid side chain (Mok and Mok, 2001; Sakakibara, 2006). More than 40 species of natural cytokinins have been identified in plant tissues (Hoyerova, *et al.*, 2006). Since endogenous cytokinin concentrations are very low (less than 50 pmol/g of fresh weight) (Novak, *et al.*, 2003), and plant tissue extracts contain complex multicomponent mixtures, exhaustive analysis of cytokinin contents is difficult. Sensitive and selective analytical tools are required for the determination of endogenous cytokinin levels in plant tissues. Levels of cytokinins are spatially and temporally regulated. Cytokinins are abundant in immature seeds (Letham, 1994). It is generally assumed that the root tip is the major site of cytokinin

synthesis, but the cambium, the shoot apex, and immature seeds are also thought to synthesize cytokinins (Letham, 1994; Emery *et al.*, 2000). Changes in cytokinin levels in association with plant development have been reported (Morris *et al.*, 1993; Benkova *et al.*, 1999; Dewitte *et al.*, 1999; Emery *et al.*, 2000; Yang *et al.*, 2001; Jacqmard *et al.*, 2002). Changes in cytokinin levels are also associated with the cell cycle, the levels being highest in the late S phase and during the M Phase (Redig *et al.*, 1996b). Environmental factors also affect cytokinin levels, which are generally positively correlated with level of mineral nutrients, especially nitrogenous nutrients (Samuelson and Larrison, 1993; Takei *et al.*, 2001b; Sakakibara and Takei, 2002), and decreased by water stress (Yang *et al.*, 2001). The levels of active cytokinins in plants are expected to be regulated by the rates of biosynthesis, inter-conversion, transport, and degradation. Cytokinins accelerate chloroplast differentiation (Harvey *et al.*, 1974) and stimulate chlorophyll production (Fletcher *et al.*, 1973) in cucumber cotyledons. This response has been used as a bioassay for cytokinins (Fletcher and McCullagh, 1971a). The African yam bean (*Sphenostylis stenocarpa* Hochst. ex. A. Rich) is a leguminous crop belonging to the family Fabaceae, sub-family papilionoidae, tribe Phaseoleae, sub-tribe Phaseolionae and genus *Sphenostylis* (Okigbo, 1973; Allen and Allen, 1987). It is grown as a minor crop in mixed

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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). Currently, there is no detailed research activity on abscisic acid and its implication in seed dormancy of *Sphenostylis stenocarpa*. Therefore, the objective of this work is to report the methanol-chloroform-formic acid-water extraction and quantification of Cytokinin from ungerminated and germinated seeds of *sphenostylis stenocarpa*.

## MATERIALS AND METHODS

**Extraction of Cytokinin:** One hundred grams (100g) of powdered seeds were macerated with 300 ml of methanol –chloroform- formic acid-water (12:5:1:2 v/v) for 24 hours at 4°C to inactivate any phosphate (Parker and Letham, 1973). It was then homogenized in the same solvent and centrifuged at 4, 000 r.p.m. for 30 minutes at 4°C. The supernatant was kept while the pellet was extracted 3 times with methanol-water-formic acid (60:40:1 v/v) at 4°C for 12 hours with continuous stirring. It was filtered under vacuum and with the combined filtrate and supernatant was reduced *in vacuo* at 35°C to an aqueous fraction (10 – 20ml). An equal volume of ethanol: water (1:1v/v) was

added to the aqueous fraction and adjusted to pH 3.4 for column chromatography.

**Polyvinylpyrrolidone (P.V.P) Column Chromatography:** The P.V.P. slurry was prepared as for gibberellins and abscisic acid. It was packed in a column (60 x 3.0cm) to a height of 30cm, and equilibrated with 0.1M phosphate buffer (pH 8.0). The extract was applied to the bed of the column and eluted with 500ml of the phosphate buffer. A 350ml fraction was collected and adjusted to pH 8.0 and partitioned three times against ethyl acetate. The basic ethyl acetate fraction (200ml) obtained was reduced to dryness *in vacuo* and the residue dissolved in an aliquot of 35% ethanol for TLC.

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 solvent was then added to give the toluene ethyl acetate/water ternary azeotrope. This was then reduced to dryness and the residue dissolved in an aliquot of 35% ethanol for subsequent TLC. The aqueous fraction from the last partition was adjusted to pH 2.5 and partitioned three times against n-butanol fraction (200ml). This was reduced to dryness and the residue was dissolved in an aliquot of 35% ethanol for TLC, bioassay and HPLC.

**Thin Layer Chromatography for Cytokinins:** Thin layer glass plate (20x 20cm) of 0.5mm thickness were prepared by using silica gel and allowed to dry at room temperature. The plates were activated at 105°C for 30 minutes. Cytokinins containing sample were loaded on the plate allowed to dry 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 105°C for 10 minutes and the resulting cytokinin bands were made visible under UV light at 254nm (yellow bands). The bands made visible under the UV light were marked and then scraped from the plates into aluminium foil for cucumber cotyledon greening bioassay (Yu *et al.*, 2006; Fletcher *et al.*, 1982).

**Cucumber Cotyledons Bioassay for Cytokinins:** The method of Fletcher *et al.*, (1981) was used. Cucumber (*Cucumis sativus* L.) seeds were planted in small poly bags and germinated in the dark at 28°C. The cotyledons from 7 day old plants were excised in dim green light, making certain that the hypocotyl hook was removed. The cotyledons were placed in 5cm Petri dishes containing various concentrations of the eluates from the chromatograms, distilled water and

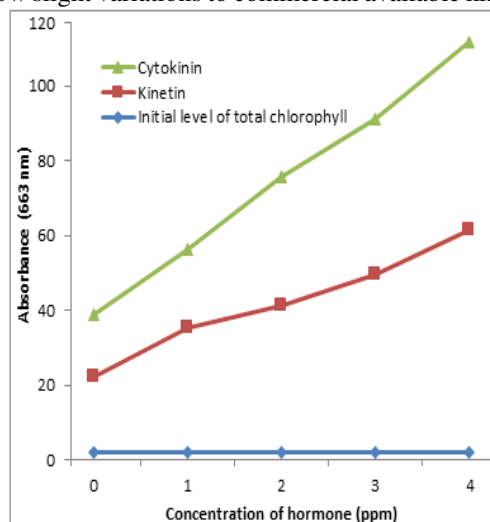
kinetin (6-furfuryl-aminopurine). The dishes were returned to the dark at 28°C for 24 hours and then exposed to two 1000 watt bulbs in a cupboard. After 3 hours, the cotyledons were homogenized, and the chlorophyll was extracted in 8ml 80% acetone. The volume was brought up to 10ml with acetone and then centrifuged at 2,500g for 10 minutes. The absorbance of the supernatant were read at 663 and 645nm. Chlorophyll content was expressed as absorbance at 663nm (Fletcher *et al.*, 1981).

**Separation and Quantification of the Cytokinin by High Performance Liquid Chromatography (HPLC):** The modified method of Basalah (2001) was adopted. The extract was purified using the Sep Pak C<sub>18</sub> cartridge. After the elution of the cytokinins from the cartridge, the eluate was concentrated with the nitrogen stream. The residue was dissolved in the 0.50 ml of 50% ethanol and filtered on the column of millipore HV 0.45 micrometers. The concentrated extract injected into the supersphere RP- select B analytical column attached to the waters 600E Multisolvant Delivery system. Elution was performed with 70% acetonitrile as solvent A and 2% acetic acid as solvent B. The applied flow rate was 0.8ml per minute at a temperature of 30°C.

## RESULTS AND DISCUSSION

**Cucumber Cotyledons Greening Bioassay for Cytokinin:** Cytokinin –like substances obtained from *S. stenocarpa* were found to induce chlorophyll biosynthesis in cotyledons of dark germinated cucumber seeds. The chlorophyll content was

proportional to the concentration of the cytokinin-like substances in the medium (Figure 1). The results show slight variations to commercial available kinetin.



**Fig 1:** Increase in chlorophyll levels in etiolated cucumber cotyledons treated with various concentrations of cytokinin-like substances and kinetin

**Analytical Quantification of Cytokinin fraction by High Performance Liquid Chromatography (HPLC):** Cytokinin composition of ungerminated and germinated *S. stenocarpa* seed as determined by High Performance Liquid chromatography is presented in Tables 1 and 2. Identification and quantification of cytokinin fractions are shown in Figures 2 to 7. Four endogenous cytokinins Zeatin (Z), trans-zeatin (TZ), Isopentenyladenine (iP) and isopentenyladenosine (iPR) were identified and quantified.

**Table 1:** Cytokinin composition of germinated seed of *S. Stenocarpa*

| Hormone fraction              | Z         | TZ      | IP      | IPR     |
|-------------------------------|-----------|---------|---------|---------|
| Basic ethyl acetate fraction  | 467.14811 | 7.08149 | 7.70569 | 2.51481 |
| Acidic ethyl acetate fraction | 533.10245 | 8.15429 | 1.04392 | 3.43576 |
| n-butanol fraction            | 662.77661 | 9.75090 | 1.24620 | 3.90485 |
| Petroleum ether fraction      | -         | -       | -       | -       |
| N-hexane fraction             | -         | -       | -       | -       |

Data shown have been computed from Figs. 2-4

**Table 2:** Cytokinin composition of ungerminated seed of *S. Stenocarpa*

| Hormone fraction              | Z          | TZ        | IP      | IPR     |
|-------------------------------|------------|-----------|---------|---------|
| Basic ethyl acetate fraction  | 1734.43622 | 21.66201  | 1.27199 | 5.69390 |
| Acidic ethyl acetate fraction | 1766.46545 | 22.443521 | 1.31820 | 6.94277 |
| n-butanol fraction            | 1960.52480 | 32.53761  | 1.62072 | 9.98202 |
| Petroleum ether fraction      | -          | -         | -       | -       |
| N-hexane fraction             | -          | -         | -       | -       |

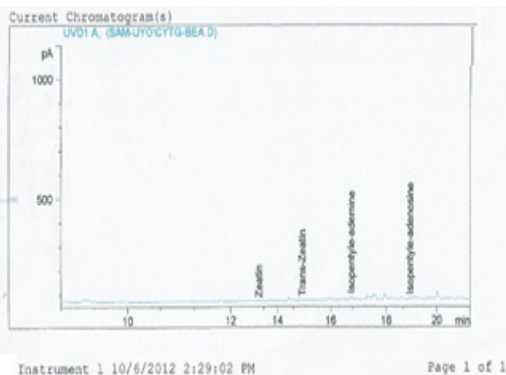
Data shown have been computed from Figs. 5-7 : **Key:** Z= Zeatin; TZ= Trans-zeatin; IP= Isopentenyl-adenine; IPR= Isopentenyl-adenosine

Cytokinin –like substances obtained from *Sphenostylis stenocarpa* were found to induce chlorophyll biosynthesis in the dark-germinated cotyledons of cucumber. The level of chlorophyll formation was proportional to the concentration of the cytokinin –like substance in the medium. There were

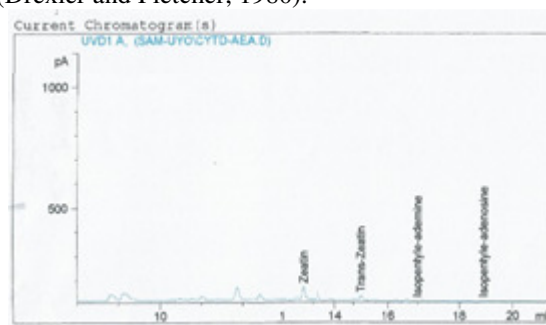
slight variations in the results when compared to the effects of commercially available kinetin. The results obtained during the bioassay with the dark-germinated cotyledons of cucumber showed the presence of cytokinin-like substances in the extracts of *S. stenocarpa*. Previous studies have established that

cytokinins accelerate chloroplast differentiation (Harvest *et al.*, 1974) as well as regulate and stimulate chlorophyll production in etiolated cucumber cotyledons (Fletcher and McCullagh, 1971; Fletcher *et al.*, 1973). The increase in chlorophyll production was found to be proportional to the concentrations of cytokinin, and this response provides a sensitive, yet rapid, bioassay for cytokinin (Fletcher and McCullagh, 1971).

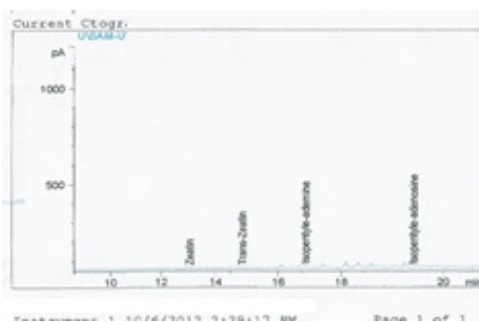
(1976) found that the cucumber cotyledon greening bioassay (Fletcher and McCullagh, 1971) was convenient and sensitive, and it provided a more linear response than did either the oat-leaf yellowing test (Thimann and Sachs, 1966) or the soybean callus test (Miller, 1965). Since then, the cucumber cotyledon assay has been used for studying the effects of herbicides, such as S-ethyl dipropylthiocarbamate, which stimulates (Weinberg and Castelfranco, 1975), and Fluoridone, which inhibits chlorophyll production (Drexler and Fletcher, 1980).



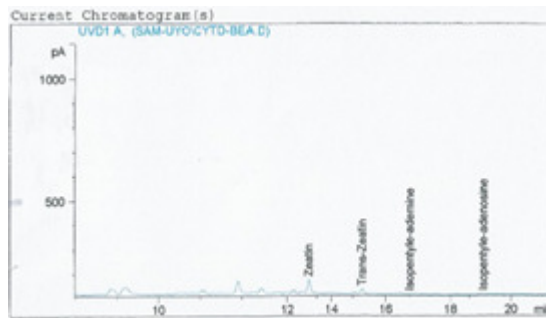
**Fig 2:** HPLC analysis of basic ethyl acetate fraction of cytokinin in germinated seed of *S. stenocarpa*



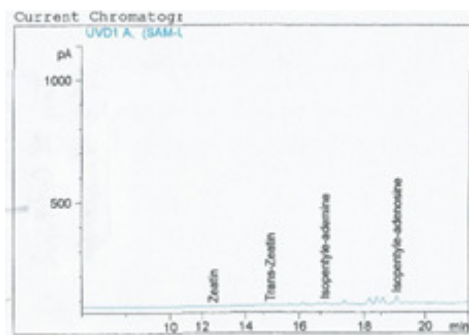
**Fig 5:** HPLC analysis of acidic ethyl acetate fraction of cytokinin in ungerminated seed of *S. stenocarpa*



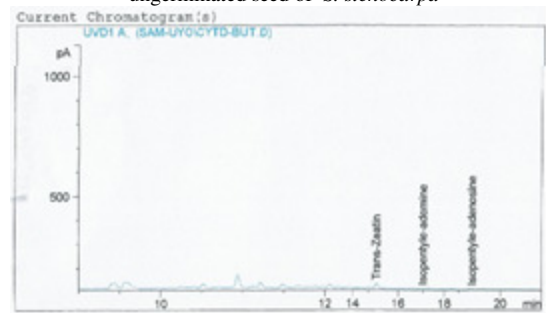
**Fig 3:** HPLC analysis of n-butanol fraction of cytokinin in germinated seed of *S. stenocarpa*



**Fig 6:** HPLC analysis of basic ethyl acetate fraction of cytokinin in ungerminated seed of *S. stenocarpa*



**Fig 4:** HPLC analysis of acidic ethyl acetate fraction of cytokinin in germinated seed of *S. stenocarpa*



**Fig 7:** HPLC analysis of n-butanol fraction of cytokinin in ungerminated seed of *S. stenocarpa*

The sensitivity and specificity of several cytokinin bioassays, including those based on leaf senescence and callus formation, have been reviewed by Letham (1987). In a comparative study, Dumbroff and Brown

Brenner *et al.*, (1976) found that the cucumber greening assay (Fletcher and McCullagh, 1971) provided a precise method for cytokinin quantification and identification. This bioassay in conjunction with chemical assays (HPLC) is used routinely for the confirmation of biological activity (Carnes *et al.*, 1973). The amount of chlorophyll produced by

etiolated cucumber cotyledons after exposure to light is dependent on the age of the cotyledons (Fletcher and Poku, 1976) as well as on the dark incubation period (Dei, 1978). The cucumber greening assay for cytokinins is being used in several laboratories. The cultivar of cucumber does not seem to be an important factor. In this study, the time of exposure to light, temperature at which the seeds are germinated, and the age of the plants (Fletcher and McCullagh, 1971) are important factors in detecting cytokinin concentrations below 1.0 ppm. An optimal age of plants for a maximum response to cytokinin was determined. In this study, 7-day-old cotyledons were used. The increase in chlorophyll was found to be proportional to the log of the concentration of cytokinin. The role of potassium in enhancing the response to cytokinin is not clear. Speculations as to its possible regulatory role as an osmoticum, an enzyme cofactor, or a non-specific promoter of metabolism are discussed by Green and Murr (1978) and Elliot (1979). The method employed was very simple, quick and inexpensive. The bioassay proved that this product can be used as an alternative to commercial grade kinetin in terms of its ability for chlorophyll biosynthesis. This extract many contain other cytokinin-like substances such as zeatin, 2R-ribosyl zeatin, 2G-glucosyl zeatin etc. (Hutton and Van Staden, 1982). In the cucumber cotyledon greening system, cytokinins eliminate lag phase (Fletcher and McCullagh, 1971b), accelerates chloroplast differentiation and stimulates enzyme activity and chlorophyll production (Harvey *et al.*, 1974).

**Conclusion:** The amount of chlorophyll produced by etiolated cucumber cotyledons after exposure to light is dependent on the age of the cotyledons as well as on the dark incubation period. In this study, the development of a sensitive and selective method for extraction, identification, characterization and quantifying cytokinins using high performance liquid chromatography (HPLC) has been described.

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