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HPLC-MS Evaluation of Mannuronic and Guluronic Acid in Bacterial Alginate from *Azotobacter vinelandii* and the Effect on Glyceollin Induction and Accumulation in Soybeans

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Aims: The objective of this study was to investigate the eliciting efficacy of bacterial alginate which was extracted from *Azotobacter vinelandii* in the induction of glyceollins and accumulation in soybeans.
Methodology: The preparative high performance liquid chromatography (HPLC) was used to detect, separate and purify the alginate after extraction and ultra performance liquid chromatography – mass spectrometry (UPLC-MS) was used to detect the molecular weights of both the extracted alginate and the induced glyceollins in soybeans.
Results: Bacterial alginate induced glyceollins synthesis in soybean seeds in high quantity. The optimal conditions of elicitation were as follows: concentration of bacterial alginate 60 µL presoaked in the sterile water for 5h, incubated at a controlled temperature of 30℃ and in the dark for 4 days.

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Alginate induction ability exhibited glyceollin-inducing activities of 1.775 (mg/g dry weight) while the control had no record of glyceollin induction. The results demonstrated that a larger molecular weight or a higher G/M ratio might correlate with a higher glyceollin-induction activity.
Conclusion: In conclusion; alginate could be introduced as relatively safe and efficient elicitor of high glyceollin accumulation in soybeans. The method for bacterial alginate extraction and its application in soybean seeds for glyceollin induction may be of practical use in the food.

Keywords: Glyceollins; alginate; elicitation; accumulation and Azotobacter vinelandii.

1. INTRODUCTION

"Soybeans are a major part of the Chinese diet and have become popular in other parts of the world as well". "Today, soybean farmers use innovative farming strategies and technology to plant new varieties that will yield healthier crops, while taking up technology and environmentally sustainable practices to protect the land for future use, also meeting the growing demand for high-quality soy proteins" [1]. "Soybean production will continue to multiply, with increasing global demands for the use of soybeans in industrial applications. The popularity of soybeans is associated with their health-promoting properties, which include reduced risks of cancer and cardiovascular disease" [2]. "These health benefits result from the presence of bioactive compounds such as isoflavones in soybeans" [3]. "Glyceollins, a major family of phytoalexins, are important isoflavones in soybeans" [4]. They have many human health benefits, such as preventing breast and ovarian carcinoma [4] preventing hyperglycemia and insulin resistance [5] and regulating lipid and carbohydrate metabolism [6]. Glyceollins accumulate in soybeans in response to various elicitors, including substances of pathogen origin (biotic elicitors), such as cell walls and spores [7- 9] and abiotic elicitors, such as heavy metal salts and chemicals [10- 12].

"However, those elicitors are either complex microorganic mixtures or poisonous compounds, which may impact the consumption of induced soybean products because of food biosafety considerations and restrict their application in the food industry" [13]. "Alginates are marine oligosaccharides generated from alginate. They contain guluronic acid (α-L-guluronate, G) and mannuronic acid (β-D-mannuronate, M) units linked by 1 → 4 *O*-glycoside bonds; these units are arranged in homopolymeric G blocks, M blocks, and random heteropolymeric G and M blocks" [14, 15]. "To date, the structure of alginate has been elucidated using ESI–MS, NMR spectroscopy and ESI–MS techniques" [16-18]. Alginate oligosaccharides have multiple roles and can be used as growth factors to stimulate the VEGF-mediated growth and migration of human endothelial cells [19, 20] and to regulate plant developmental and defensive processes [21- 25]. "However, there are very few studies on the induction capacity of alginate for glyceollin accumulation in plants, especially soybeans" [26]. "In our previous research, we reported the glyceollin inducing activity of alginate in soybeans; however, the alginate used as elicitors were a mixture of sodium alginate oligomers" [27]. "The identification of alginate derived from the M-, G-, or MG-blocks and their sequence determination are known to be important for better understanding of the structure–function relationships of alginates at the molecular level" [28]. "In the food industry, alginates are widely used as additives capable of viscosifying, stabilizing/emulsifying, and gelling aqueous solutions" [29, 30]. "More than half of the total alginate produced worldwide is used in food industries such as ice cream, icy custards, cream and cake mixtures, as well as to keep the contents in suspension in fruit juices" [31]. "Although, seaweeds are the major source of alginate for commercial use, however, the alginate produced by the bacteria is considered to be of good quality as compared to that obtained from the algae. The bacterial alginate has better qualitative properties, based on its chemical structure similarity compared to those of algal alginate and it is more cost effective compared to algae" [32]. "Two bacterial genera, *Azotobacter* and *Pseudomonas* have been extensively studied to produce alginate. *Azotobacter vinelandii* is the most preferred alginate producer strain for large scale production, based on its safety, chemical structure similarity compared to those of algal alginate and it is more cost effective compared to algae" [33]. "Moreover, the alginate obtained from *Pseudomonas* has poor jellifying ability" [34]. "On the other hand considerable pathogenic ability associated with species of *Pseudomonas* has made *Azotobacter* the favorite genus for alginate production" [35]. "However, most of the

studies about alginates have focused on the chemical characterization and physicochemical properties, such as chemical structure, molecular weight, gelation behavior, and ability to interact with other polymers" [36]. There are few articles involving in the elicitation ability of alginate for soybean phytoalexins induction, especially for phytoalexin glyceollins. This research detailed herein, is the first to report on the efficient induction of soybean glyceollins elicited by bacterial alginate which was extracted from *Azotobacter vinelandii*.

2. MATERIALS AND METHODS

2.1 Materials

Soybeans (*Glycine max),* which have an exceedingly high level of isoflavones variety were purchased in sufficient quantity from Abakaliki International market in Ebonyi State. The glyceollin standard was prepared in the Laboratory of the Federal Institute of Industrial Research FIIRO, Oshodi, Lagos State by making use of the preparative high performance liquid chromatography (HPLC), the method adopted was according to Eromosele et al. [37]. HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Only HPLC-grade water was used to prepare reagent solutions. All other analytical-grade reagents were purchased from Beijing Chemicals Reagent Company (Beijing, China). (UPLC–MS), the solvent acetonitrile used was purchased from fisher scientific company (Fair Lawn, NJ, USA).

2.2 Methods

2.2.1 *Azotobacter vinelandii* and culture conditions

The parent strain of *Azotobacter vinelandii* ACCC-10087 was purchased from the culture bank of Agricultural Culture Collection of China (ACCC). The strain was kindly provided in lyophilized form by ACCC culture collection (Beijing, China). The lyophilized cells were activated first in yeast mannitol agar, (YMA) medium comprised of the following composition (g/L): Mannitol, 10.0; yeast extract, 1.0; NaCl, 0.1; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2 and H_2O 1000mL. The pH of this medium was adjusted to 6.8 before sterilization. The inoculated cultures were placed in an incubator shaker (Zhicheng, China) at 28°C and 180 rpm for 24 h to ensure cell viability. After 24 h, 0.1 mL of this suspension was aseptically transferred to Burk's nitrogen free agar medium slants. The slants were placed in an incubator (Santn, China) at 28°C for 24-72 h. After the bacterial growth, the slants were stored at 4°C in a cool cabinet (Sanyo, Japan). The grown colonies were harvested in 50 % glycerol solution (v/v) and subsequently stored in cryo-vials for cell banking at -20˚C to minimize the productivity loss by subsequent cultivations of cells. Each experiment started by revival of one glycerol vial in vegetative culture [38].

2.2.2 Alginate extraction and separation

After incubation, the alginate produced by *Azotobacter vinelandii* was extracted with an improved method as described by Byrd et al. [39] with some modifications. 50mL of the culture after incubation period of 96h was mixed with the volume of (1M) NaCl and then centrifuged at 12,000 rpm for 30 min. The supernatant was removed and the volume of 2% cetyl pyridium chloride was added. Cetyl pyridium chloride was added to enable precipitation of alginate in the solution, after which the extraction of alginates was achieved by centrifugation at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was kept. 50 mL of NaCl (Sodium chloride) was added to the pellet to dissolve it and the same volume of cold Isopropanol was also added and then mixed well. The addition of NaCl and Isopropanol were used to break the alginate-calcium complex to facilitate removal of bacterial cells. After centrifugation, the supernatant was removed and the pellet was again resuspended in 50 mL of saline, (0.85% NaCl). The pellets with larger amount of alginates were allowed to completely resuspend. It was then kept overnight at 4˚C for further analysis and soybean seed treatment.

2.2.3 HPLC and spectrophotometric conditions for bacterial alginate determination

The alginates were analyzed using a Waters Alliance series HPLC system, a Waters 2996 photodiode array detector, a Waters HPLC workstation and a TSK-GEL DEAE-2SW 4.6 mm×250 mm column (Tosoh Corp., Tokyo, Japan). Method used was according to Takamitsu *et al* with some modifications. Elution was carried out at a flow rate of 1.5 mL/min with the following solvent system: A= 0.25M NaCl; B = double distilled H_2O. The temperature was set at 35°C, monitored wavelength was at 230 nm by

a UV detector. Bacterial alginate was determined by using a spectrophotometric method adopted by Brivonese and Sutherland [34], Conti et al. [40] with some modifications. This method was used because it is sensitive, accurate and independent of alginate composition. Reading of the optical density (O.D) of cells was measured by Biomate 3S UV spectrophotometer (Thermo, Scientific Co., shanghai, China) at 550 nm.

2.2.4 Separation of alginate by HPLC

The alginate sample (1g) was mixed with 100 ml of water for 2 h in room temperature. The mixture was centrifuged at 15,000 g for 5 min, and the supernatant was filtered through a 0.22 lm sterile syringe filter. The filtered extract was applied as a crude alginate solution. 1 ml sample was fractionated and collected by a Sykam S1125 preparative HPLC system equipped with an S3245 UV detector operating at a wavelength of 230 nm, Clarity ver. 7.1 software and a TSKgel DEAE-2SW preparation column (20.0 mm I.D. 9 25 cm, Tosoh Corp., Tokyo, Japan) using a 180 min linear gradient of 0–0.25 M NaCl and a flow rate of 7.0 ml/min.

2.2.5 Purity test on alginate fractions

The collected alginate fractions were checked on analytical HPLC Waters 2695 system equipped with a 2996 UV detector and Empower Pro software under previously described conditions by Chaki et al. [41] Column, TSK DEAE-2SW 4.6 mm I.D. 9. 25 cm (Tosoh Corp., Tokyo, Japan); column temperature, 30^{o}C; monitor wavelength, 230 nm; mobile phase, water-0.25 M NaCl with a linear gradient (0–60 min); flow rate, 1.0 ml/min; injection amount, 20 µl; analytical time, 60 min. The purity of each fraction was calculated via the HPLC area normalization method.

2.2.6 (ESI-MS) mass spectrometry analysis of samples

“ESI–MS was conducted in negative-ion mode with a Waters Xevo TQ-s instrument (Waters Corp.). Each sample was dissolved in water and diluted in 50% aqueous methanol. The ESI–MS conditions were as follows: injection volume, 2 µl; ESI voltage, 4 kV; capillary temperature, 275 ^{o}C; capillary voltage, 350 V; tube lens, 250 V; scan range, 400–2000 m/z. Nitrogen was used as the sheath gas and auxiliary gas at a flow rate of 30 and 5 arb, respectively. The mobile phase (methanol/water = 1:1, v/v) was delivered at a flow rate of 200 µl /min” [47].

2.2.7 Glyceollin induction

“Glyceollin induction in soybean cotyledons was performed according to the method described by Boue´ with some modifications” [2]. “The Alginate mixtures were dissolved in water to provide 1% (w/v) solutions. Each solution (60 µl) was applied to the cut surface of a soybean cotyledon. In the control group, water (60 µl) was applied. All chambers were sealed with parafilm and incubated for 4 days at 25 ^{o}C in the dark” [2].

2.2.8 Glyceollin determination

“Soybean cotyledon extraction and sample preparation were carried out according to the method described by Eromosele in our previous research” [37]. The accumulation of glyceollins was calculated by the following formula: $y = 1.0 \times 10^{7} + 754.32$ ($R^2 = 0.9999$).

3. RESULTS AND DISCUSSION

3.1 Separation of Bacterial Alginate by HPLC

Bacterial alginates extracted from *Azotobacter vinelandii* in this study were successfully detected and separated on an anion-exchange column by preparative HPLC (Fig. 1a). This analytical method yielded a series of distinct and well-resolved peaks indicating the presence of guluronic and mannuronic acids which were defined as (G and M). Results obtained show that guluronic and mannuronic (G and M) acid fractions of the alginate standard samples were obtained by hydrolysis as seen in (Fig. 1a and 1b) which had two peaks fraction. The retention times recorded were as follow: (7.14 min) for guluronic and (8.23 min) for mannuronic. All the fractions were collected and analyzed further via analytical HPLC as shown in Fig. 1b and 1c. The relative purities of these fractions mainly reached a high level of > 97%.

3.2 Confirmation of the Separated Alginate Fractions by (ESI-MS) Mass Spectrometry

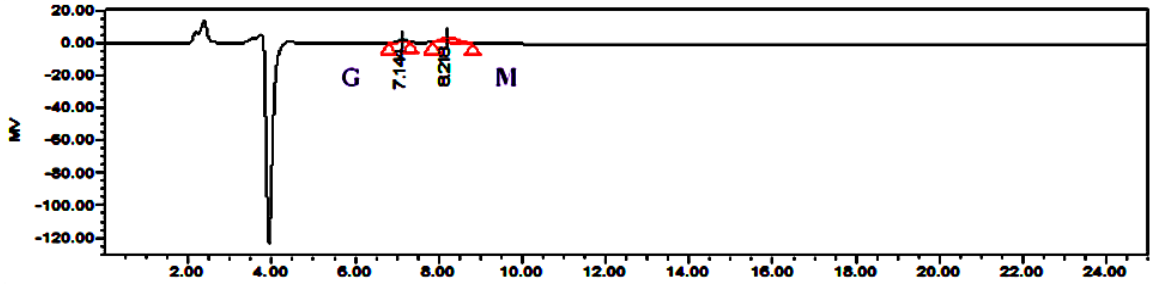
As shown in Fig. 2, the negative-ion mode mass spectra of guluronic and mannuronic acid displayed signals corresponding to the main deprotonated oligomers $[M–H]^-$ (M is the molecular weight of the main solute) and a series of ions with Na, i.e., $[M + Na–2H]^-$, $[M + 2Na–3H]^-$, $[M + 4Na–5H]^-$, etc. With the aim of improving sensitivity and reducing interferences, the two

uronic acids were also analyzed in SIR mode, selecting the 193 and 175 m/z fragments. The result shows the signals corresponding to α -L-guluronic acid and β-D-mannuronic acid in chromatograms obtained by successive direct injection, in SIR mode were 175 m/z and 193 m/z. Fig. 2 shows that the two fragments differed in abundance: in β-D-mannuronic acid the 174 m/z fragment was less predominant, while in α-L- guluronic acid the 174 m/z fragment and the 193 m/z fragment were present in similar amounts, and in mannuronic acid the 193 m/z fragment was predominant. The results obtained for the UPLC-ESI-MS is in agreement with the result obtained by Zhang et al. [42].

3.3 Glyceollin Induction and Determination

The glyceollin induction activity of treated and un-treated soybean seeds after treatment with alginate as elicitor for a period of 4 days incubation in the dark is shown in (Fig. 3).

To determine the glyceollin-induction activities of bacterial alginate in this study with G/M ratios, the glyceollin concentration in soybean cotyledons was calculated by HPLC analysis. As shown in Fig. 4a, b and c glyceollins were synthesized after induction by the bacterial alginate. Glyceollins showed peaks with retention time of 23–24 min, which corresponded



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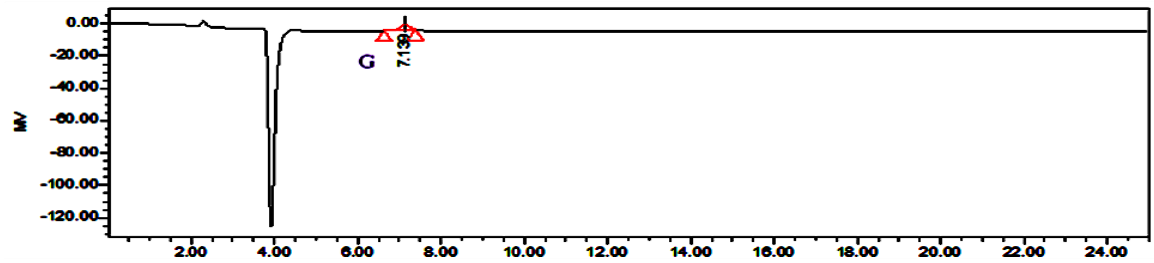

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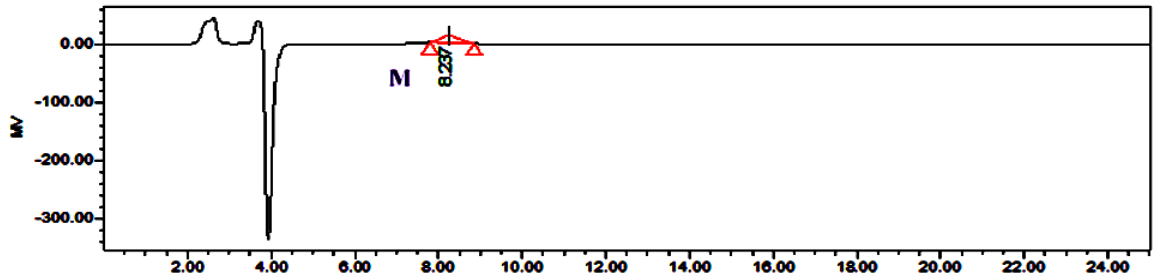


(1c)

Fig. 1. (a) HPLC profile of bacterial alginate sample having guluronic and mannuronic (G and M) components which was extracted from *Azotobacter vinelandii*. (b) Preparative HPLC profile of separated β-guluronic acid (G) from bacterial alginate sample. (c) Preparative HPLC profile of separated D-mannuronic acids (M) from the alginate extracted from *Azotobacter vinelandii*

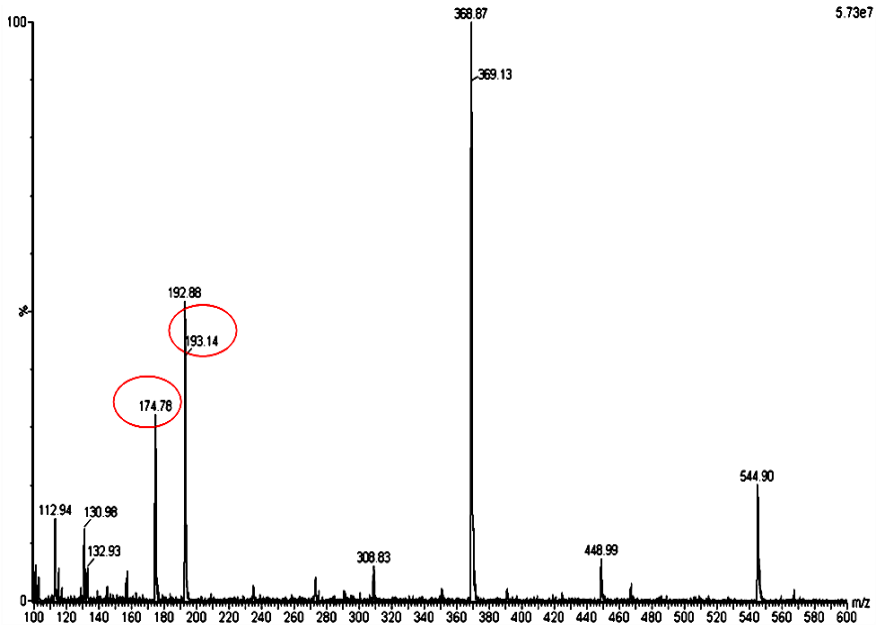
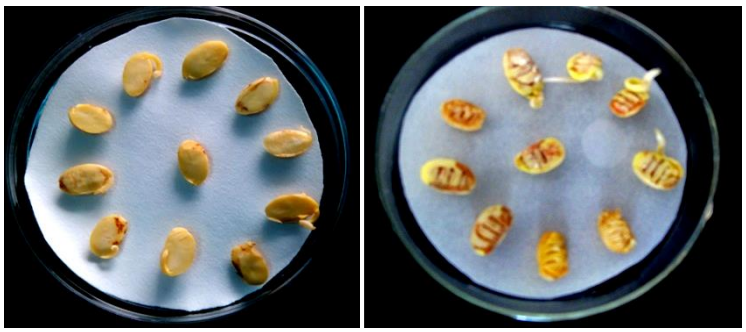


Fig. 2. (ESI-MS) mass spectra of guluronic and mannuronic (G and M) fractions which were collected by preparative HPLC. The mobile phase used during the mass spectrometry analysis are (methanol / water = 1:1, v/v) which was delivered at a flow rate of 200 µl/min



A **B**

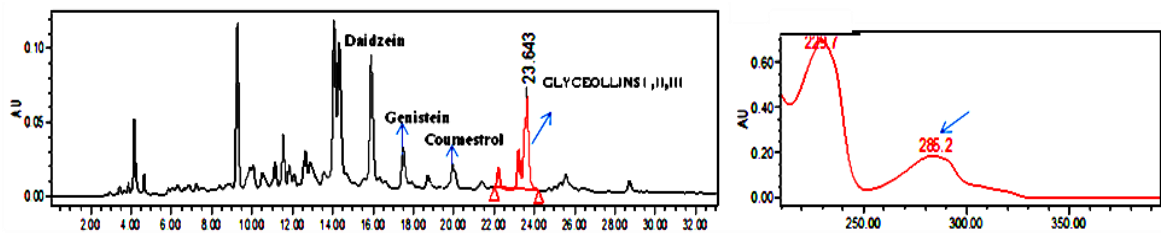
Fig. 3. (a) un-treated soybean seeds (b) treated soybean seeds after incubation in the dark for 4 days. The treated soybean seeds induced glyceollin upon treatment with the bacterial alginate. The colour change witnessed was as a result of the presence of phytochemicals induction

to the presence of glyceollin I, II and III as recorded by Jenkins et al. [2]. The concentration of glyceollins was calculated according to the method described by Eromosele et al. [37]. Results obtained shows that bacterial alginate used as elicitor yielded (1.775 mg·g-1 dry weight) of glyceollin following optimal conditions derived in this study. Meanwhile, the alginate standard sample from seaweed source was only able to produce glyceollin in lesser amount as quantification recorded (0.04 - 0.12 mg·g-1) which was low when compared with the accumulation of glyceollins induced with the bacterial alginate. The induction of glyceollin increased in accumulation when bacterial alginate was applied as elicitor in soybean seeds.

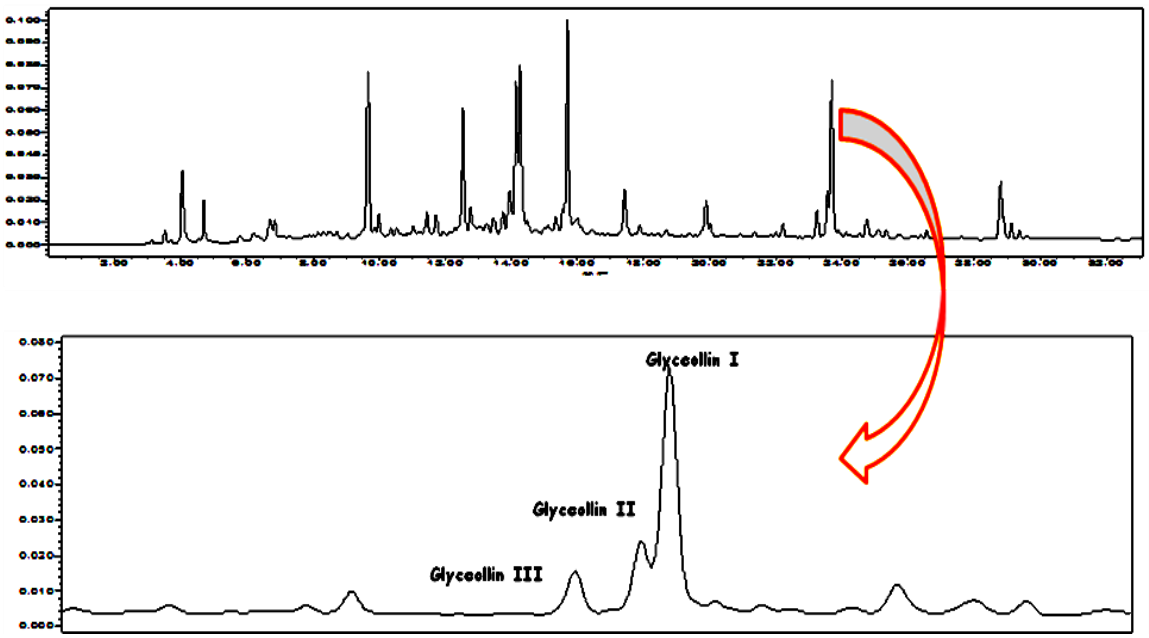
3.4 Results of UPLC-MS Analysis of Glyceollin Elicited by Bacterial Alginate

The UPLC-MS method was validated in terms of sensitivity and retention run time. The major advantages of the UPLC over the HPLC were the fast analysis, narrow peaks, high sensitivity and reduction of solvent consumption. In the present study, the UPLC method was used for the

analysis and identification of glyceollins. The results from the UPLC-MS analysis chromatogram shows that individual separated peaks of the sample component were analyzed, and the desired phytochemical of interest (glyceollin) was detected in the bacterial alginate treated soybean seeds. Glyceollin retention time was recorded at 3.77 and 8.87 min, see (Fig. 5a) when UPLC was carried out for the analysis of glyceollins at m/z 339.



(4a)



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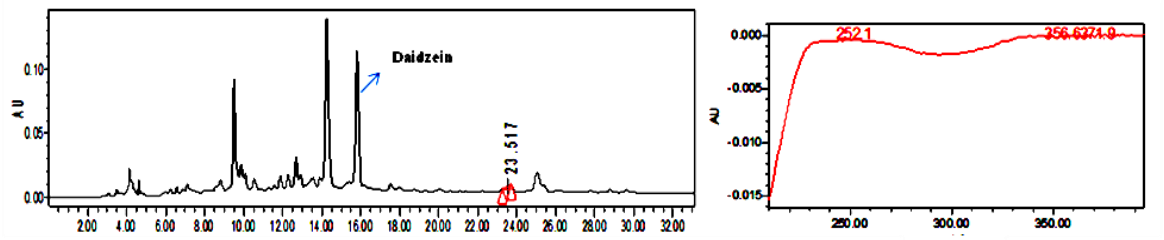

(4c)

Fig. 4. The HPLC results of un-inoculated and inoculated soybean seeds: (a) HPLC chromatogram of bacterial alginate treated soybean seeds detailing the induction of glyceollins (I, II and III). (b) HPLC chromatogram of bacterial alginate enlarged chromatogram identifying the three isomers of glyceollins. (c) HPLC chromatogram of healthy soybean seeds without elicitor. All The chromatograms were obtained by recording wave length UV absorbance at 285 nm

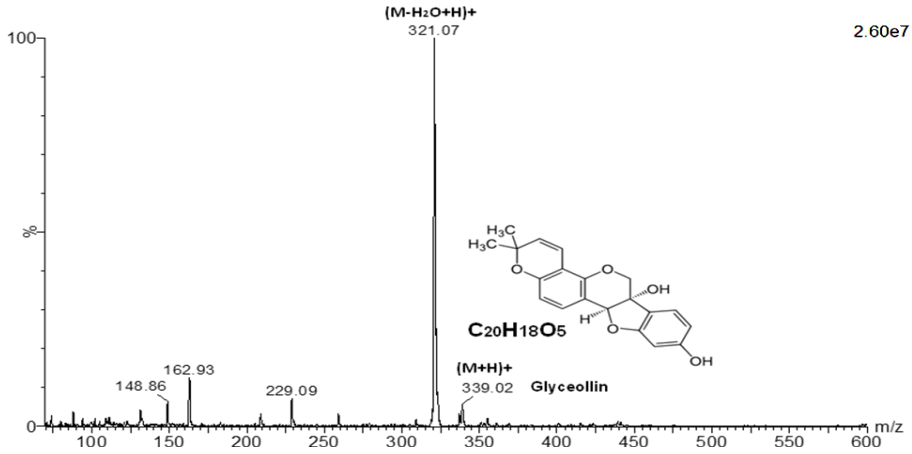

Fig. 5a. Positive ion electro-spray ionization mass spectrometry analysis of glyceollins isolated from soybean seeds treated with bacterial alginate

3.5 Confirmation of Glyceollin by Electro Spray Ionization (ESI-MS) Mass Spectrometry

The positive ion ESI mass spectrum of glyceollin is shown in (Fig. 5b). The glyceollin induced in this study was confirmed by electro spray ionization (ESI) mass spectrometry. The base peak in the spectrum is the ion at m/z 321 detailing the loss of one water molecule ($M - H_2O+H)^+$ and the molecular ion at m/z 339 represents protonated glyceollin $(M + H)^+$. Similar mass spectra were obtained for glyceollin extracted from this study which is in agreement with the molecular formula ($C_{20}H_{18}O_5$). The application of the UPLC-MS method was utilized in this study for the identification of glyceollin, and this was achieved in a shorter chromatographic run time when compared with the HPLC. It was conducted to determine the molecular weight of the phytochemical glyceollin in soybean seeds treated with the bacterial alginate extracted from *Azotobacter vinelandii*.

3.6 Discussion

In this research, the presences of α-L-guluronic and β-D-mannuronic acids (G and M) were determined in the alginate extracted from *Azotobacter vinelandii* by making use of the analytical methods of the HPLC and ESI-MS technology. The extracted alginate was used for the first time as elicitor for glyceollin induction in soybeans to determine the activity of alginate as an elicitor of metabolites. It was observed that changes in the amount of isoflavones in both inoculated and un-inoculated soybean seeds were monitored and the isoflavonoid of interest glyceollins were quantified for maximum accumulation based on each peak area of the HPLC chromatogram. Alginate extracted in this study had increased eliciting glyceollin activities in soybeans (Fig. 4a). Such a similar weight-activity relationship was also observed for the antimicrobial activity of chitosan according to [42] and the growth promotion activity in lettuce seedling roots of pectate oligosaccharide mixtures by [23]. Moreover, as shown in Fig. 4b, we found that alginate with a higher G/M ratio induced a higher glyceollin concentration in soybeans; this indicated that the glyceollin-inducing activities are related not only to their molecular weight but also to their chemical structures, such as the G/M ratio. There was no peak recorded for glyceollin induction by HPLC chromatogram for the control sample according to (Fig. 4c). This finding correspond with the report by Ku¨pper, who found out that alginate oligomers with a higher G/M ratio could elicit a higher reactive oxygen species level in the sporophytes of the kelp Laminaria digitata [43]. "In plants, reactive oxygen species are important in signaling cascades and are continuously produced as byproducts of various metabolic pathways involved in the plant immune system" [44]. "Glyceollins are the main type of secondary metabolites in soybeans and play crucial roles in plant microbe interactions" [45, 46]. "Therefore, we propose that the alginate with a higher G/M ratio induce a higher reactive oxygen species level in soybean cotyledons, which may ultimately correspond to a stronger signal for the glyceollin synthesis pathway. To confirm this hypothesis, further studies, as well as large amounts of alginate with different structures, are recommended for further research" [47].

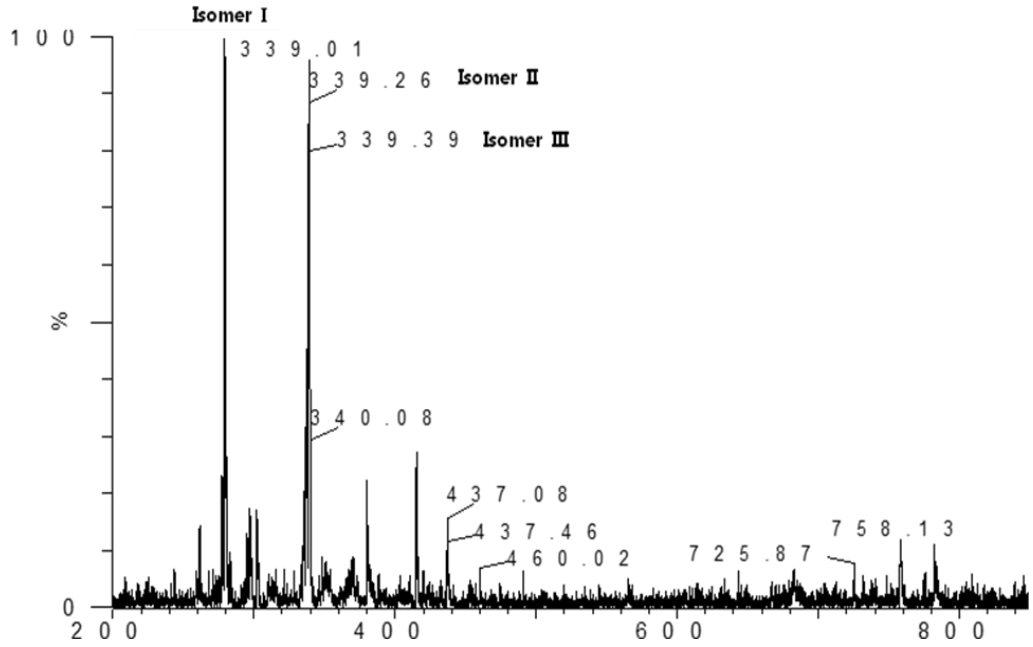

Fig. 5b. UPLC-MS chromatogram of glyceollin (a). The chromatogram shows the individual separated peaks of the sample; glyceollin was detected in soybean seeds treated with bacterial alginate. (b) The resolution of the positional isomers of phytochemical glyceollins (I, II, III)

4. CONCLUSION

To the best of our knowledge, this is the first report on the use of bacterial alginate extracted from *Azotobacter* exhibiting induction capacities of glyceollin accumulation in soybean seeds. Alginate with a higher G/M ratio induced a higher glyceollin concentration in soybeans as observed in this study. Alginate has become an elicitor which can induce glyceollin biosynthesis in soybeans alongside other elicitors like *Aspergillus sojae*, *Aspergillus niger*, chemicals like silver nitrate which has been reported in previous studies by different authors. Moreover, the method of alginate production, extraction and application described here may be of practical use in the food industry to develop nutraceutical products with high antioxidant capacity. It is therefore suggested that alginate could be introduced as a relatively safe and efficient elicitor in the induction of glyceollins in soybean seeds, since its application has been in use in the food industry.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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