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(54) Title: METHODS FOR INDUCING ANTHOCYANIN PRODUCTION IN PLANTS

(57) Abstract: Protocols are described to elicit high anthocyanin expression in plants by controllable and predictable methods. Methods of producing and using anthocyanins are described. Also described are methods for eliciting anthocyanin production in plants having beneficial properties for growth and re-harvest that do not usually produce anthocyanins in useful quantities.



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TITLE

METHODS FOR INDUCING ANTHOCYANIN PRODUCTION IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to United States Provisional Application Number 61/723,043, filed on November 6, 2012, the entire disclosure of which is expressly incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

[0002] In the food coloring and pigment industry, there is a need for natural pigments and anthocyanins are highly sought after compounds. These pigments range in color from red to purple to blue depending on molecular functional groups and pH, and are in demand for not only their color but also for their health promoting properties. These molecules are responsible for a variety of plant colors and also possess numerous beneficial attributes that range from anti-cancer therapies to helping advance solar energy technology.

[0003] Currently, only a limited number of plants are used as sources to extract these molecules for human use. A majority of plants used for commercial anthocyanin production can only be harvested once or twice per year, are costly, and do not express coloration across the entire plant. Examples of plants known for their anthocyanin production include grape, blueberry, raspberry, chokeberry, hibiscus, purple corn, and red cabbage. In order to meet present and future demand, new species need to be examined as sources for anthocyanins.

[0004] There is a need for alternative efficient techniques for anthocyanin production. Using plants that are efficient to grow, and that have leaf and stem anthocyanin expression, tissue could be harvested multiple times per month or growing season. There is a need for selecting species with favorable traits, for: growth, re-harvest, and anthocyanin expression in leaf tissue. There is also a need for protocols to elicit higher anthocyanin expression by controllable and predictable methods.

[0005] There is no admission that the background art disclosed in this section legally constitutes prior art.

SUMMARY OF THE INVENTION

[0006] In a first broad aspect, there are provided methods for increasing anthocyanin production in a plant, a method comprising: selecting a plant; cultivating the plant; applying stress to the plant, wherein the stress comprises: subjecting the plant to light stress comprising: intense

synthetic light and long duration light exposure; harvesting plant tissue; and repeating the cultivating, applying stress, and harvesting. The cultivating may optionally include fertilizing the plant. In some embodiments, a fertilizer may be applied during the cultivation or recovery period and withheld during the period of stress. In some embodiments, the steps of cultivating, applying stress, and harvesting are repeated twice, three times, four times, or five or more times.

[0007] In another aspect, there is provided a method of plant treatment for the elicitation of anthocyanin production in a plant, wherein a majority of the above-ground plant tissue is harvested at least once in a single growing season, the treatment comprising: cultivating the plant; applying stress, wherein the stress comprises: subjecting the plant to light stress comprising: intense light and long duration light exposure; harvesting the majority of the above-ground plant tissue; and extracting anthocyanins from the tissue.

[0008] In some embodiments, the yield of anthocyanin is increased by 50-200% as compared with a control plant, of the same variety, grown under standard conditions. In some embodiments, the yield of anthocyanin is increased by 100-500%, 200-700%, 500-1000%, 1000-10,000%, and in some embodiments, the yield of anthocyanin is increased by as much as 12,000%.

[0009] In some embodiments, the elicitation produces 50-250mg/100g fresh weight of anthocyanins in foliage in a plant that produces less than 5mg/100g fresh weight of anthocyanins in foliage under standard growing conditions.

[0010] In some embodiments, the plant is stressed by light stress comprising white light. In some embodiments, the plant is stressed by light stress comprising blue light. In some embodiments the light is continuously applied. In some embodiments, the stress comprises light stress comprising a light duration of 24 hours/day for at least 3 days. In some embodiments, the duration is 3-8 days. In some embodiments, the stress comprises light stress of sunlight in combination with synthetic light, wherein total instantaneous photosynthetic photon flux is approximately $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. In some embodiments, the light stress comprises continuous exposure to blue light, at an intensity of $400\text{-}1500 \mu\text{mol m}^{-2} \text{s}^{-1}$, for four to seven days. In some embodiments, the duration is 8 days. In some embodiments, the duration is approximately 5 days. In some embodiments, the light stress comprises blue light wherein the wavelength is approximately 450-495nm. In some embodiments, the light stress comprises white light having a measured instantaneous photosynthetic photon flux of approximately $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. In some embodiments, the light stress comprises white light having a high measured instantaneous photosynthetic photon flux. In some embodiments, the high measured instantaneous photosynthetic photon flux within a range of $400\text{-}1500 \mu\text{mol m}^{-2} \text{s}^{-1}$, $900\text{-}1,200 \mu\text{mol m}^{-2} \text{s}^{-1}$, approximately $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, or $1,200\text{-}2,000 \mu\text{mol m}^{-2} \text{s}^{-1}$. In some embodiments the light is cycled between light and dark in a short period. In some embodiments, a light source comprises a high intensity discharge (HID) lamp. In some embodiments, a light source comprises a light-

emitting diode (LED).

[0011] In some embodiments, the invention is embodied in a plant treated by a method described. In some embodiments, the plant is red cabbage. In some embodiments, the plant selected is a turfgrass. In some embodiments, the plant is *Poa trivialis*. In some embodiments, the plant is *Agrostis stolonifera*. In some embodiments, the plant is selected from the group consisting of the plants listed in Table 9, Table 10, Table 11, and Table 12. In some embodiments, the plant selected is not transgenic.

[0012] In some embodiments, the stress further comprises osmotic stress. In some embodiments, the stress further comprises osmotic stress, wherein the osmotic stress is selected from the group consisting of: withholding water; treatment of foliage with a film-forming anti-transpirant; application of abscisic acid; and application of one or more osmolytes, wherein the osmolyte is sugar, salt, fertilizer, bi-carbonate, protein, or a water soluble organic molecule. In some embodiments, the stress further comprises osmotic stress, wherein water is not applied for a period of greater than 24 hours.

[0013] In some embodiments, the stress further comprises a growth medium comprising 75-100% sand.

[0014] In some embodiments, the stress further comprises a growth medium low in phosphate.

[0015] In some embodiments, the stress further comprises cool temperature. In some embodiments, the stress further comprises a growing temperature between 0-15°C. In some embodiments, the growing temperature is between 5-20°C, 5-10°C, -5-10°C, or 0-20°C.

[0016] In some embodiments, the majority, by weight, of harvested plant tissue comprises leaves. In some embodiments, the plant selected produces less than 1% dry weight anthocyanins under standard growing conditions, wherein standard growing conditions are conditions optimal for biomass production and biomass yield, and wherein the plant produces 2-20% dry weight anthocyanins under the methods described. In some embodiments, the plant yield of anthocyanin is greater than 1 g/kg dry weight.

[0017] In some embodiments, greater than 50% of the anthocyanin produced is cyanidin 3 malonyl-glucoside.

[0018] In another aspect, there is provided an ornamental plant in which a red or purple coloration is elicited by the application continuous bright light, at 400-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, for 30 to 100 hours at a temperature of 0-15°C, wherein the elicitation produces 20-200mg/100g fresh weight of anthocyanins in foliage and wherein the plant produces less than 15mg/100g fresh weight of anthocyanins in foliage under standard growing conditions.

[0019] In another aspect, there is provided a photoprotective composition comprised of anthocyanins, wherein the composition is adapted for application to reduce stress in plants. In

some embodiments, the composition is used for plants exposed to high levels of ultraviolet radiation and/or visible light. In some embodiments, the composition further comprises: a sticker and a carrier. The composition may further include dyes, pigments, pesticides, fertilizers, and fungicides.

[0020] Various objects and advantages of this invention will become apparent to those skilled in the art from the following detailed description of the preferred embodiment, when read in light of the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] **FIG. 1:** A structural depiction of an anthocyanin. Functional groups may include but are not limited to: hydrogen, hydroxyl, or methoxy. Sugars can be attached to one or more positions, typically R₃, and these sugars can be substituted with several types of acyl groups or other sugars. Functional groups may include other constituents.

[0022] **FIG. 2:** Biosynthesis of anthocyanins as controlled by phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS). Both enzymes are intensely controlled by the given environment especially blue light and high light intensity; however, many species are unique for the specific combination of stress to induce anthocyanin synthesis.

[0023] **FIG. 3:** Chromatogram and mass spectra breakdown from rough bluegrass anthocyanin analysis. Two major peaks were identified: peak 1 = cyanidin 3 – glucoside and peak 2 = cyanidin 3 – malonyl glucoside; minor peaks were not identified. These anthocyanins represent molecules present following elicitation and not present prior to elicitation.

[0024] **FIG. 4:** A depiction of the overall anthocyanin elicitation scheme on a turfgrass plant. Step 1: the application of stress on a young or mature turfgrass plant, for example, high intensity white light or blue light. Step 2: leaf tissue is mechanically harvested, removing a majority of the tissue. Step 3: fertilizer is applied and adequate water is supplied. In this step, fertilizer should be applied at a rate of no less than .10 lbs. nitrogen/1000 ft², and should be around 1 lb. on average. Adequate water must be applied on a regular interval. Step 4: healthy tissue is re-grown for a period of at least 1 week. Following re-growth, the entire protocol can be re-run on the same plant rather than starting again from seed.

[0025] **FIG. 5:** A comparison of total monomeric anthocyanin concentration for rough bluegrass sown in soil-less media elicited with HID light 4 times. There was no difference in rough bluegrass elicited for the first time compared to plants elicited past their first time. This shows that rough bluegrass is not negatively affected by elicitation; therefore the same plants can be elicited over again without a decrease in anthocyanin yield.

[0026] **FIG. 6:** A comparison of total monomeric anthocyanin concentration for rough bluegrass sown in 100% sand elicited with HID light 4 times. There was no difference in rough

bluegrass elicited for the first time compared to plants elicited past their first time. This shows that rough bluegrass is not negatively affected by elicitation; therefore the same plants can be elicited over again without a decrease in anthocyanin yield.

[0027] **FIG. 7:** A comparison was made between data from experiment 1 and experiment 2 to determine if the type of soil had an effect on anthocyanin concentration. Results show that rough bluegrass sown in 100% sand had an average total monomeric anthocyanin concentration of 235.12 mg/100g, and rough bluegrass sown in soil-less media had an average total monomeric anthocyanin concentration of 138.80 mg/100g. These results were statically significant ($P = .05$), showing that rough bluegrass sown in a sandy soil will produce greater concentrations of anthocyanins.

[0028] **FIG. 8:** Results of experiment 3A, show similar trends to previous experiments. Rough bluegrass did not show a decline in the ability to produce high concentrations of anthocyanin following the first elicitation. Effectively, continuous blue light did not have an adverse physiological effect that prevented further elicitations.

[0029] **FIG. 9:** Results of experiment 3B show the same trend as experiment 3A. Rough bluegrass did not show a decline in the ability to produce high concentrations of anthocyanin following the first elicitation. Continuous blue light did not have an adverse physiological effect that prevented further elicitations. Experiments 3A and 3B exhibit the repeatability of using blue light as an induction source.

[0030] **FIG. 10:** A comparison of anthocyanin production using high intensity discharge (HID) lamps and LED lights. Data for HID lamps is presented as the average total monomeric anthocyanin production from experiment 1 and 2, and data using LED lights is the average concentration for experiments 3A & 3B. Both are also compared to the average total monomeric anthocyanin concentration in non-elicited rough bluegrass. Rough bluegrass exposed to HID light produced statically greater ($P = .05$) concentrations of anthocyanins; therefore, this light treatment also showed a larger percent increase compared to plants not elicited. When HID lights were used light intensity was $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ on average; however, light intensity of the blue LED array was approximately half of this value. This trend effectively shows that increasing light intensity has the ability to increase anthocyanin concentration, even though there is not a specific wavelength being used.

[0031] **FIG. 11:** When comparing total monomeric anthocyanin concentration for 5 cultivars of rough bluegrass, it was found that there was no statistical difference ($P = .05$) between cultivars in their ability to induce high concentrations of anthocyanin. Therefore, 'Havana' is not unique in this aspect.

[0032] **FIG. 12:** When comparing the ability to up regulate anthocyanin production, one cultivar showed a significant difference ($P = .05$). 'ProAm' showed the largest percent increase

compared to the other cultivars; however, these results do not have an overall impact relative to overall concentration.

[0033] **FIG. 13:** Anthocyanins from red cabbage are typically extracted from mature cabbage heads. This experiment exposed young red cabbage to blue light to evaluate if it had the ability to increase anthocyanin concentration at this stage of maturity. Results showed that red cabbage exposed to blue light for 6 days increased anthocyanin concentration by 166%. This combination of species and protocol increases the availability of valuable anthocyanins.

[0034] **FIG. 14:** Variation in accessional anthocyanin content is shown. The numbers on the right of each bar indicate the cultivar Accession I.D. as shown in **Table 13**.

[0035] **FIG. 15:** Variation in accessional anthocyanin content is shown with groupings and indicating the cultivar Accession I.D. as shown in **Table 13**. The low grouping includes I.D. numbers 20, 2, 11, 14, 1, and 18. The transition grouping includes I.D. numbers 13, 3, 15, 10, 9, 6, 5, 19, 4, 12, 17, and 16. The high anthocyanin grouping includes I.D. numbers 24, 7, 23, 22, 25, 21, and 8.

[0036] **FIG. 16:** Shown are molecular precursors relevant in anthocyanin biosynthesis.

[0037] **FIG. 17A:** Mesophyll thickness is shown. The numbers under each bar indicate the cultivar Accession I.D. as shown in **Table 13**. least significant difference (LSD) = 8.5738. As shown, two varieties producing more anthocyanin have thin mesophyll.

[0038] **FIG. 17B:** An image from an electron scanning microscope is shown for a sample of leaf from Accession I.D. 15 of **Table 13**.

[0039] **FIG. 17C:** Leaf cross-sections are shown. Top left panel shows Accession I.D. 8 at magnification x150. Top right panel shows Accession I.D. 25 at magnification x200. Bottom left panel shows Accession I.D. 1 at magnification x120. Bottom right panel shows Accession I.D. 15 at magnification x150.

[0040] **FIG. 18A:** A graph of cuticular wax is shown relative to anthocyanin concentration. As shown, plants producing more wax produce less anthocyanin. $R^2=0.4187$, $P=0.0431$ ($\alpha=0.05$).

[0041] **FIG. 18B:** A comparison of cuticular wax content is shown for ten varieties. The numbers under each bar indicate the cultivar Accession I.D. as shown in **Table 13**. LSD=2.4158.

[0042] **FIG. 19A:** Pre-existing phenolic content and induced anthocyanin concentration is shown.

[0043] **FIG. 19B:** Pre-existing flavonoid content and induced anthocyanin concentration is shown.

[0044] **FIG. 20A:** Percent increase in phenolics following 48 hours of treatment is shown.

[0045] **FIG. 20B:** Percent increase in flavonoids following 48 hours of treatment is shown.

[0046] **FIG. 21:** Exogenous applications of synthetic pigments compounds have the ability to filter and reflect both excess visible light and UV light. To determine the effects of applications of

pigment green 7, varying concentrations of the pigment were applied to a creeping bentgrass.

Results show a dose dependent effect.

[0047] **FIG. 22:** Creeping bentgrass plants were treated with either a 400 PPM rough bluegrass anthocyanin extract, or solution of green 7 (16 oz./Acre, approximately 1,000 PPM) to determine their effects on reducing photochemical stress. Results show that over a period of 27 hrs. under high light intensity both green 7 and the anthocyanin spray exhibited a trend of increasing photochemical efficiency compared to an untreated control (UTC).

* UTC = B, Anthocyanin = B, Green 7 = A

**UTC = B, Anthocyanin = AB, Green 7 = A

***UTC = B, Anthocyanin = A, Green 7 = A

[0048] **FIG. 23:** Total chlorophyll content is shown for creeping bentgrass comparing pre-treatment plants to plants stressed under high light intensity light over a period of 27 hours and untreated, sprayed with anthocyanin and sprayed with green 7.

[0049] **FIG. 24:** To evaluate possible detrimental effects on chloroplast photosystems both total chlorophyll content (**FIG. 23**) and the chlorophyll *a:b* ratio (**FIG. 24**) were examined. Following light treatment, results showed that both chlorophyll parameters responded in the same manner for all three treatments. The data shows that there were no negative physiological effects of the anthocyanin spray at 400 PPM.

[0050] **FIG. 25:** Pigments can be sprayed on plant foliage to help reflect and absorb potentially damaging light while still allowing the transmission of photosynthetically active radiation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0051] Anthocyanins (**FIG. 1**) are plant pigments produced by secondary metabolism that have uses in many applications. The majority of research in anthocyanin production has been focused on fruiting plants. Unlike many plant products, plant stress can increase anthocyanin yield. Although, plants have been known to produce anthocyanins under adverse conditions, earlier attempts at controlled elicitation have not been successful or reliable.

[0052] Described herein are methods for using whole plants, or a majority of above-ground tissue, rather than fruits or seeds, to produce anthocyanins. Whole plants exposed to abiotic and/or biotic stress can produce high concentrations of anthocyanins in a relatively short amount of time. High intensity white light or blue light can provide the major source of stress. Following anthocyanin accumulation, tissue is harvested and used for suitable applications. Furthermore, using specific species, such as varieties of turfgrass, allows the ability to “re-use” the same plant to produce anthocyanins again rather than starting the whole process over by seed.

[0053] Anthocyanins are either produced constitutively or are induced by specific environmental conditions. When anthocyanins are continually produced, many species will

exhibit slow growth and lower yield. To combat this and yet still maintain fitness, plants have developed mechanisms to produce anthocyanins under stress.

[0054] Phenylalanine-ammonia lyase (PAL) catalyzes the first committed step in anthocyanin biosynthesis (**FIG. 2**). Regulation of this enzyme is controlled by numerous environmental factors; therefore, anthocyanin production is also regulated by the environment. Relative to other events, regulation of PAL by light has been the most studied. Two photoreceptors, phytochrome and cryptochrome, have been noted to positively regulate PAL; however, their involvement is overlapping and complicated. For phytochrome, red light and far-red light have been shown to regulate the transcription of PAL genes; however, the wavelength that ultimately regulates PAL is dependent on both the given species and the specific tissue. A second photoreceptor, cryptochrome, has also been shown to be a key regulator. Rather than sense light quality, like phytochrome, cryptochrome perceives light quantity in the form of blue light. The classic cryptochrome response is a high irradiance response (HIR), where high light intensities stimulate a signal transduction cascade that results in gene transcription. Ultra-violet light, namely UV-B, is also known to increase the transcription of PAL, either via cryptochrome or through an un-named UV photoreceptor.

[0055] Beyond being controlled by light, PAL and anthocyanins are also regulated by water status/potential, nutrient status, wounding, senescence, cold temperature, and pathogen elicitation. PAL is a key enzyme in anthocyanin synthesis, but there are multiple other enzymes that directly regulate the production of anthocyanins.

[0056] Chalcone synthase (CHS) catalyzes the first reaction of flavonoid biosynthesis using the products from the phenylpropanoid pathway and intermediates from fatty acid synthesis. Just like PAL, CHS is environmentally regulated (**FIG. 2**). However, unlike PAL, both UV-B and blue light serve as the primary sources of light induced CHS transcription. This enzyme has also been shown to be indirectly regulated by sucrose, linking photosynthetic activity, possibly source/sink relations, or water potential. Hormones also act as positive regulators of CHS, where gibberellins induce its transcription in *Petunia hybrida*. In newly developing tissue, it's not rare to see a high concentration of anthocyanins, and this may be in part due to increased hormonal activity as well the tissue being a sucrose sink.

[0057] Both PAL and CHS are intensely regulated by the given environmental conditions, tissue/organ type, and plant maturity. One or multiple environmental signals may serve to initiate the transcription of all the necessary enzymes of anthocyanin synthesis. The regulation of anthocyanin synthesis is very complicated. Depending on the maturity of the tissue, the type of tissue, or the current environmental conditions anthocyanins will only accumulate if they are needed in many plants. For most plants, these are not constitutive compounds; therefore, their regulation needs to be complex in order to only produce them when necessary. Anthocyanins are

induced under stressful situations to help protect the plant from events that may be physiologically harmful. Purposely applied stress can be used to artificially promote plants to produce anthocyanins in higher quantities. To further improve anthocyanin yield, specific plants must be used. Plants that produce anthocyanins that are in demand can be stressed at a given maturity to increase anthocyanin concentration or to manipulate quantities of various types of anthocyanins. Artificial stress can also be applied to species that rarely produce anthocyanins in order to produce overall larger quantities per year. In order to fully take advantage of anthocyanin stress induction, species should meet specific qualifications.

[0058] Plants can be divided into perennial and annual types. Perennial plants survive for multiple years, whereas annuals will only live through one season. Using perennial plants allows the ability to use the entire plant multiple times for anthocyanin production rather than re-starting from seed. Furthermore, plants that propagate themselves through manners other than seed are also highly suited to be anthocyanin sources. These plants will spread vegetatively and can tolerate tissue removal allowing for biomass harvesting throughout a season. Perennial plants that have the ability to tolerate persistent tissue harvesting are perfect examples of plants that can be used as potential sources of anthocyanins. Turfgrasses are a prime example of a group of species that express many of the necessary attributes for anthocyanin stress induction. These plants have a growing point at or below the soil surface that allow rapid tissue regeneration following tissue harvesting. This is the primary reason they can be mowed over and over again without dying. Many turfgrasses also readily spread clonally by stolons, allowing for an even quicker recovery following mowing.

[0059] In order to serve as a source of anthocyanins, plants must reliably produce anthocyanins under the given stress protocol. Previous work only using exposure to high levels of ultra-violet (UV) irradiation proved to be a poor means for anthocyanin elicitation (Nangle, Edward J., "Ultraviolet light and its effect on germination, growth, physiology and pigment responses of cool season turfgrasses." Electronic Thesis, Ohio State University, 2012). Creeping bentgrass, tall fescue (*Schedonorus arundinaceous*), and perennial ryegrass (*Lolium perenne*) were exposed to high dose UV-B (313 nm) light for up to 7 days. All species showed little to no ability to repeatedly produce anthocyanins; however, 4 anthocyanin molecules were identified in creeping bentgrass, but concentration was too low for quantification. Rather than directly stimulating anthocyanin production, UV light can serve as a supplemental stress when combined with primary sources of light stress: high intensity white or blue light.

[0060] Anthocyanins can be induced by a number of factors; however, it's the combination of a specific species with specific factors that ultimately results in production of high anthocyanin concentrations. Rough bluegrass (*Poa trivialis*) is a turfgrass species that is identified by the presence of a purple sheath. This species is relatively weak, and succumbs to environmental

stress. However, rough bluegrass is also very vigorous and can recover from stress quickly once the stress is removed. This species constitutently produces very low concentrations of anthocyanin (C.V. 'Havana', $\leq 1.5\text{mg}/100\text{g}$ – **FIG. 10**, rough bluegrass prior to elicitation), but it will drastically up regulate under extreme stress. Using high intensity white light or blue light, this species has the ability to increase anthocyanins production by upwards of 12,000% (**FIG. 10**). The following experiments are examples of anthocyanin stress induction. High intensity white light or blue light served as the primary source of stress, while any other environmental stress was considered supplementary.

[0061] **EXAMPLES**

[0062] Certain embodiments of the present invention are defined in the Examples herein. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

[0063] *Experimental materials and methods, Experiments 1-5*

[0064] All plants were grown in the same greenhouse. Plants were hand watered daily, fertilized bi-weekly (1 lb. nitrogen/1000 ft²) using Peters Professional® 20-10-20, and periodically treated with pesticide. Imidacloprid N-[1-[(6-Chloro-3-pyridyl) methyl]-4,5-dihydroimidazol-2-yl]nitramide (Bayer Environmental Science, Research Triangle PK, NC) was preventively and post-emergently applied for insect control. Mefenoxam (R,S)-2-[(2,6-dimethylphenyl)-methoxyacetyl amino]-propionic acid methyl ester (Syngenta AG, Greensboro, NC) and thiophanate-methyl (dimethyl 4,4'-o-phenylenebis[3-thioallophanate]) (Cleary Chemicals, Dayton, NJ) were used for fungi control and prevention. Environmental conditions in the greenhouse were setup as follows: 15 hour photoperiod, total light threshold of 300 watt/m² (supplemental lighting was turned on when solar radiation dropped below 300 watt/m²), daytime cooling: 74°F (23°C), daytime heating: 70°F (21°C), nighttime cooling: 67°F (19°C), and nighttime heating: 63°F (17°C).

[0065] *Experiment 1: Established plants stressed by high light, cool temperature, and low water.*

[0066] Six-inch pots of rough bluegrass ('Havana') that had been previously growing under greenhouse conditions in soil-less media were evaluated for anthocyanin production when exposed to high intensity white light for a prolonged period of time. Light treatments were imposed within a Conviron E15 growth chamber (Controlled Environment Ltd., Winnipeg, Canada) equipped with high intensity discharge (HID) lamps. Two magnetically driven metal halide ballasts (Phillips

Advance) producing 400 watts each at 120 volts were mounted to the exterior of the growth chamber to reduce heat. Wiring and light sockets were passed into the chamber to allow mounting of the lamps. Lamps were hung on separate laboratory ring stands to allow for height adjustment. The growth chamber was programmed to run for 24 hours at a temperature of 15°C with no other parameters being adjusted. Four rough bluegrass plants were randomly arranged underneath the metal halide lamps into 2 blocks, each lamp being a block, and distance from the lamps was adjusted to achieve a given irradiance. Plants were exposed to irradiances between 900-1,200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 hours (77.8 – 103.7 $\text{mol m}^{-2} \text{day}^{-1}$) for a total time of 5 days. Past 5 days there was no visual increase in anthocyanin production; however, intervals less than or greater than 5 days could be used. During this period water was withheld to promote drought. Drought stress was considered to be a supplementary stress for this protocol. Once drought stress was visually noticeable, plants were watered daily until the end of the protocol.

[0067] Drought stress could be initiated by means other than withholding water. The addition of some compound that results in a reduction of soil water potential could also be used to initiate drought. These compounds could be soluble carbohydrates (i.e. sucrose), salt solutions, protein solutions, etc. Drought stress could also be promoted by hormone application. Applications of abscisic acid (ABA) would result in stomatal closure; therefore initiating a water stress response. Many permutations in this stress protocol could be used to enhance anthocyanin production; however visible light stress serves as the backbone where other stress factors enhance the protocol.

[0068] Tissue was harvested down to 1-2 cm from the soil surface, was snap frozen in liquid nitrogen, and was lyophilized for 24 hours. Snap freezing is essential to avoid enzymatic and environmental degradation of the metabolites; however lyophilization is not necessary and only makes extraction relatively easier. Following sample preparation, tissues were ground in liquid nitrogen and extracted in acetone following the methods of Rodriguez-Saona and Wrolstad (2001, “Anthocyanins: Extraction, isolation and purification of anthocyanins” Current Protocols in Food Analytical Chemistry). The liquid extraction was then used to determine total monomeric anthocyanin concentration by the pH differential method. To determine the anthocyanins produced by rough bluegrass under stress, the extraction was further processed and analyzed using LC-ESI MS (Liquid chromatography – Electron Spray Ionization Mass Spectrometry). The extract was purified by means of C-18 column chromatography following the methods of Rodriguez-Saona and Wrolstad (2001), and was suspended in .01% acidified DI water for analysis.

[0069] The following LC-MS (Shimadzu Scientific Instruments) setup was used for anthocyanin identification. A CTO-20A oven was used to maintain temperatures at 35°C. A flow rate of 0.8 mL min^{-1} was controlled with LC-20AD – LC pumps, while 20 μL of extract was injected using a SIL-20AC autosampler. Samples were injected onto a Varian C18-A, 121 150 x 4.6 mm i.d. column (Varian Inc.). Samples were eluted through a SPD-M20A photodiode array

(PDA) with wavelengths 280, 320 and 520 nm selected. Wavelength absorption between 250 and 700 nm was also recorded for all peaks. A binary flow mobile phase consisting of (A) 4.5% formic acid/H₂O (v/v) and (B) acetonitrile with a flow rate of 0.8 mL min⁻¹ was used.

Anthocyanins were separated using the following solvent gradient: 12% B 1-25 minutes, 35% B 25-30 minutes, a 35%-12% decrease in B from 30-38 minutes, and 12% B until minute 45.

Samples were then injected into an electron spray mass spectrometer and fragmentation analysis was carried out. Results showed that rough bluegrass contained two primary anthocyanins following elicitation: cyanidin 3- glucoside and cyanidin 3- malonyl glucose (**FIG. 3**). Relative molecular amounts were not quantified.

[0070] Total monomeric anthocyanin concentration was shown to be 129.40 mg/100g (all concentrations in freeze dried tissue) on average for this first experimental run (Table 1 – Time 1). However, in order to determine if the whole plants could be induced again the same four six-inch pots of rough bluegrass were exposed three more times under the same protocol. Following initial tissue harvest, plants were returned to un-stressful greenhouse conditions, were well watered, and fertilized at a rate of 1 lb. nitrogen/1000 ft². Re-growth and recovery took place over approximately two weeks, and following this period plants were put under the previously used stress induction protocol (**FIG. 4**). Results showed that over the course of 4 elicitations there were no differences between elicitations (**Table 1** and **FIG. 5**). This effectively shows that elicitation does not have a negative impact on physiology following tissue harvest; therefore allowing rough bluegrass plants to be exposed multiple times with no decrease in anthocyanin yield.

[0071] **Table 1: Experiment 1**

Treatment	mg/100g	mean separation
Time 1	129.40	A
Time 2	133.12	A
Time 3	120.39	A
Time 4	172.31	A
LSD	105.75	

[0072] Statistics were performed using SAS 9.2 (SAS Institute, 2002) and the GLM procedure. Elicitations were considered treatments, and are labeled times 1-4 depending on their respective order. A randomized complete block design was used for analysis, where each HID lamp was considered a block and each lamp had two samples underneath. Fisher's least significant difference ($P = .05$) was used for mean separation. Treatments showing no significant statistical difference exhibit the same letter for their respective mean separation.

[0073] *Experiment 2: Newly seeded plants stressed by high light, cool temperature, and low water.*

[0074] This second experiment used newly seeded rough bluegrass ('Havana') rather than pre-existing turf, as was used in Experiment 1. A total of six pots were seeded with a seeding rate

of 2 lbs. seed/1000ft² into a 100% sand mix (mason's grade sand) and were allowed to grow for approximately 3 months. Plants were then exposed to the same protocol from Experiment 1 using metal halide lamps as the primary induction source. Again, these plants were harvested five days following elicitation and were allowed to re-grow, well watered and fertilized at a rate of 1 lb. nitrogen/1000 ft², in the same manner as Experiment 1. These plants were also induced on four separate occasions to determine the repeatability of the protocol.

[0075] With total monomeric anthocyanin concentration as the primary parameter, results showed that there were no differences between elicitations (**Table 2** and **FIG. 6**). Again, this effectively shows that rough bluegrass can be induced to produce high concentrations of anthocyanins, harvested and allowed to re-grow, and induced over again without any decrease in anthocyanin production. A comparison was made between data from experiment #1 and #2 to determine if the type of soil had an effect on anthocyanin concentration (**FIG. 7**). Results show that rough bluegrass sown in 100% sand had an average total monomeric anthocyanin concentration of 235.12 mg/100g, and rough bluegrass sown in soil-less media had an average total monomeric anthocyanin concentration of 138.80 mg/100g. These results were statically significant ($P = .05$), showing that rough bluegrass sown in sand will produce greater concentrations of anthocyanins.

[0076] **Table 2: Experiment 2**

Treatment	mg/100g	Mean separation
Time 1	209.10	A
Time 2	223.10	A
Time 3	240.2	A
Time 4	268.10	A
LSD	369.48	

[0077] Statistics were performed using SAS 9.2 and the GLM procedure. Elicitations were considered treatments, and are labeled times 1-4 depending on their respective order. A randomized complete block design was used for analysis, where each HID lamp was considered a block and each lamp had three samples underneath. Fisher's least significant difference ($P = .05$) was used for mean separation. Treatments showing no significant statistical difference exhibit the same letter for their respective mean separation.

[0078] *Experiment 3: Assessment of blue light for anthocyanin elicitation*

[0079] The following experiment replaced the metal halide HID lamps with blue LED's to evaluate the effects of a specific wavelength of light on anthocyanin elicitation. Blue light emitting diode (LED) arrays were constructed as follows. LED's were mounted on a 1.22m x .61m 16 gauge aluminum sheet with 3M® 8805 double sided thermally conductive tape (0.127mm thick). Blue (472nm) LED's were purchased as 5050 surface-mount device (SMD) strips (Torchstar®). Each strip contained 60 LED's per meter, consumed approximately 72 watts per

strip (12 volt DC & 250mA), and had a beam angle of 120°. All LED's were mounted on metal-core printed circuit boards (MCPCB) which allowed for individual strips to be cut as small as 5.08cm containing 3 LED's. Strips were soldered together using 16 gauge standard wire and rosin core solder.

[0080] The blue LED array consisted of 54 strips, placed together with no space between, cut at 1.17m, where each strip contained 69 individual LEDs and the array had a total of 3,726 LEDs. 4-5 strips were soldered together and received power at the beginning and end of the circuit. Each set of wired strips were considered an individual unit and were not wired with other sets to help decrease the load on power sources. 12 volt AC-DC switching power supplies (constant current LED drivers) were used to regulate power to the LED's (360 or 400 watt). A total of eight power supplies were used to avoid over-loading a power supply or under-powering LED strips.

[0081] LED arrays were mounted within a Conviron E15 growth chamber (Controlled Environment Ltd., Winnipeg, Canada) set to a constant temperature of 15°C. The array was hung underneath a pre-existing adjustable light rack with spring loaded carabiners. LED drivers were placed on top of the light rack, and LED wire leads were fed up to the power sources to be connected. This setup allowed for precise distance control, where light intensity at the canopy could be tuned by adjusting the distance between the plant canopy and the LED array. In order to avoid LED overheating, 250 CFM or greater ventilation duct blowers were placed on top of the light rack and were allowed to blow down on the LED array. Heat is efficiently transferred from the conductive tape to the aluminum sheet; however, once at the sheet forced air movement is necessary for convection to occur. Three blowers were evenly spaced above the array, and were operated continuously to decrease the temperature across the LEDs.

[0082] Rough bluegrass, 'Havana', was seeded into a 100% sand mix (mason's grade sand) at a seeding rate of 2 lbs. seed/1000ft² and was allowed to grow for approximately 3 months prior to treatment. For treatment, plants were arranged into 2 blocks (left half and right half of the array) to take into account variation in light intensity. The LED array was adjusted to maintain a distance of 2.5-3 cm from the peak height of the turfgrass canopy. Light intensity at the peak of the canopy averaged between 425-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and the array ran for a 24 hour day-length. Rather than a five day elicitation period, plants were exposed a total of seven days with a 24 hour day-length, and were watered daily to avoid drought stress (unlike experiments 1-2 where drought stress was imposed). Similar to experiments 1 and 2, plants were exposed, harvested and allowed to re-grow, well watered and fertilized at a rate of 1 lb. nitrogen/1000 ft², and were exposed two more times to determine protocol repeatability. This experiment was repeated a second time on a new set of seeded rough bluegrass plants (experiment 3B).

[0083] For both runs of this experiment (experiments 3A and 3B) results show that there were no significant differences between elicitations (**Table 3, Table 4, FIG. 8, and FIG. 9**), and there

was also no differences between experiments A and B (**Table 5**). A comparison of anthocyanin production using HID lamps and LED lights was made (**FIG. 10**). Data for HID lamps is presented as the average total monomeric anthocyanin production from experiments 1 and 2, and data using LED lights is the average concentration for experiments 3A and 3B. Both are also compared to the average total monomeric anthocyanin concentration in rough bluegrass prior to elicitation. Rough bluegrass exposed to HID light produced statistically greater ($P = .05$) concentrations of anthocyanins (186.95 mg/100g compared to 140.83 mg/100g); therefore, this light treatment also showed a larger percent increase compared to plants not elicited. When HID lights were used light intensity was $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ on average; however, light intensity of the blue LED array was approximately half of this value. This trend effectively shows that increasing light intensity has the ability to increase anthocyanin concentration, regardless of a specific wavelength.

[0084] **Table 3:** *Experiment 3A*

Treatment	mg/100g	mean separation
Time 1	129.61	A
Time 2	147.24	A
Time 3	130.37	A
LSD	77.94	

[0085] **Table 4:** *Experiment 3B*

Treatment	mg/100g	mean separation
Time 1	131.46	A
Time 2	164.89	A
Time 3	141.45	A
LSD	222.80	

[0086] **Table 5:** *Treatment comparisons with combined data from Exp. 3A & Exp. 3B[±]*

Treatment	Lower bound (mg/100g)	LS mean (mg/100g)	Upper bound (mg/100g)
Time 1	115.29	130.53	145.77
Time 2	140.83	156.07	171.31
Time 3	120.67	135.91	151.15

[±]Significant difference did not exist between experiments; therefore pooled data could be analyzed. Significant difference exists if confidence intervals overlap.

Lower bound = LS mean – SE*

Upper bound = LS mean + SE

*SE = 15.24

[0087] Statistics were performed using SAS 9.2 and the GLM procedure. Elicitations were considered treatments, and are labeled times 1-4 depending on their respective order. A randomized complete block design was used for analysis, one half of the array was one block and

the other half the second; blocks consisted of three plants per block. Fisher's least significant difference ($P = .05$) was used for mean separation. Treatments showing no significant statistical difference exhibit the same letter for their respective mean separation. To evaluate the combined effects of experiment A and B, the MIXED procedure was used rather than GLM (SAS 9.2). Experiments were treated as random factors and mean separations are made using confidence intervals.

[0088] *Experiment 4: Cultivar comparison.*

[0089] In order to determine if rough bluegrass cultivar 'Havana' was unique in its ability to up regulate anthocyanin production other cultivars were tested. Experiment 4 consisted of testing four rough bluegrass cultivars obtained from the Western Regional Plant Introduction Station (Pullman, WA) and 'Havana'. The following cultivars were used: 'Laser', 'ProAm', 'Sabre', 'Colt', and the previously used C.V. 'Havana'.

[0090] Cultivars were seeded into soil-less media and were allowed to grow for approximately three months prior to treatment. The blue LED experimental protocol was used in the same fashion as Experiment 3, and this experiment was repeated a second time following vegetative propagation of the original plants. Data was analyzed for total monomeric anthocyanin concentration and percent increase in total monomeric anthocyanin concentration. Results show that 'Havana' is not the only cultivar having the ability to up regulate anthocyanin production under stress (**Table 6** and **FIG. 11**). Cultivars showed an increase in anthocyanin production ranging from 700% up to approximately 4,700%. Only one cultivar was showed a significant difference in % increase ('ProAm') while all others were statically the same (**Table 7** and **FIG. 12**).

[0091] **Table 6: Cultivar comparison for total monomeric anthocyanin concentration[±]**

Treatment	Lower bound (mg/100g)	LS mean (mg/100g)	Upper bound (mg/100g)
'Laser'*	29.12	35.77	42.42
'ProAm'**	26.58	34.86	43.13
'Sabre'***	19.07	26.18	33.29
'Colt'***	23.19	30.30	37.47
'Havana'***	21.23	28.34	35.45

*SE = 6.65

**SE = 8.27

*** SE = 7.11

[0092] **Table 7: Cultivar comparison for % increase in total monomeric anthocyanin concentration[±]**

Treatment	Lower bound (%)	LS mean (%)	Upper bound (%)
'Laser'*	1,756.86	2,365.13	2,973.40
'ProAm'**	3,227.84	3,989.90	4,751.96

'Sabre'***	1,237.45	1,887.64	2,537.83
'Colt'***	705.18	1,355.37	2,005.56
'Havana'***	1,031.21	1,681.40	2,331.59

*SE = 608.27

**SE = 762.06

*** SE = 650.19

*Significant difference did not exist between experiments; therefore pooled data could be analyzed.

Significant difference exists if confidence intervals overlap

Lower bound = LS mean – SE*

Upper bound = LS mean + SE

[0093] Data was analyzed using SAS 9.2 and the MIXED procedure. Experimental design was setup as an incomplete randomized block design consisting of 4 blocks. Block, group, and experiment were treated as random factors and data from both experimental runs was combined for analysis.

[0094] *Experiment 5: Elicitation effect on non-grass species with high innate anthocyanin production*

[0095] All previously described stress induction protocols could be performed on species other than rough bluegrass. Rather than evaluate another species that normally produces small concentrations of anthocyanins, experiment 5 set out to evaluate a species that is known for its anthocyanin production, red cabbage. For this experiment red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*, C.V. 'Mammoth Red Rock') was stressed at a young age. Seeds were sown into soil-less media and allowed to grow for 19 days prior to elicitation. Three young plants were exposed to the blue LED protocol for 6 days, and were then harvested to determine the percent increase in anthocyanins compared to three plants that were left under greenhouse conditions. This experiment was also repeated a second time on newly seeded plants, and results are presented as the average from both experiments. Results showed an average 166.64% increase in anthocyanins compared to plants not exposed to blue LEDs (**Table 8** and **FIG. 13**). Red cabbage is an annual plant that will not re-grow once harvested; however, the anthocyanins it produces are highly valued. This experiment shows that anthocyanin induction by light stress has the ability to work on species that already produce valuable anthocyanins in significant quantities.

[0096] **Table 8:** *Comparison of elicited and non-elicited red cabbage**

Treatment	mg/100g (freeze dried tissue)	Mean separation
Elicited	1,870.50	A
Non-elicited	701.50	B
LSD	908.69	

*Results are for averaged data from both experimental repetitions

[0097] Statistics were performed using SAS 9.2 and the GLM procedure. A randomized complete block design was used for analysis, where each plant was placed under 1/3 of the LED array in order to account for variation in light intensity. Fisher's least significant difference ($P =$

.05) was used for mean separation. Treatments showing no significant statistical difference exhibit the same letter for their respective mean separation.

[0098] *Protocol Variations and Permutations*

[0099] *Light*

[00100] This elicitation protocol utilizes visible light stress. There are two primary sources of this stress: excess white light or excess blue light. Either must produce light intensities between 400-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$; however intensities outside of this range can be used with alterations in the total length of time exposed. High intensity white light or blue light can serve as the primary induction source alone. Any additional stress serves to supplement anthocyanin elicitation. The following light can be used to supplement the primary sources of light stress: UV-A, UV-B, red light, and far-red light. The sources of these wavelengths of light can include incandescent, fluorescent, electrical-gas discharge (HID), and LED lights.

[00101] Blue light may broadly encompass some wavelengths in the green or ultraviolet-A range. Blue light will have a wavelength within the range of 400-500 nanometers (nm). More preferably, blue light will have a wavelength within 450-495nm. For example, a 470 nm blue LED provides a good blue light source.

[00102] *Elicitation period*

[00103] Experiments 1-5 used various total elicitation times, ranging from a total of 5 to 7 days. These periods are dependent on the total amount of light perceived by the plant per day. Light intensities could be increased to speed the process up, or intensities could be decreased to slow the process down. Optimization for particular species and cultivars can be reached by altering the stress intensity and duration. In many instances, slightly decreasing light intensity promotes increased production by means of decreased tissue damage, but must be counterbalanced by the reduced light levels leading to poor elicitation. Elicitation could also be extended past 7 days to promote further concentration; however, this must be counterbalanced by anthocyanin degradation over time. On average, it takes between 24-48 hours for anthocyanins to be visible following initial induction; therefore elicitation times between about 5 - 7 days are the most appropriate.

[00104] *Temperature*

[00105] The temperature used for all of the previous experiments was set for a constant 15°C. This temperature is slightly below optimal air temperature for C3 photosynthesis; therefore, increasing light stress and enhancing anthocyanin production. However, ranges in temperature could be used. For C4 photosynthetic plants, temperatures falling around 0°C in combination with high light intensity are an effective inducing environment. Temperature variation between 0 - 15°C would be best, but temperature above 15°C would also be acceptable. Temperatures above 30°C would be excessive for use of the protocol with cool season turfgrass, and would not promote

efficient anthocyanin elicitation. Diurnal temperature variation is also an acceptable option. Morning, day, and night-time temperatures could fluctuate between the optimal temperatures, 0 - 15°C, not exceeding 30°C, throughout the entire day-length, or a constant temperature can be maintained.

[00106] *Water stress / osmotic stress*

[00107] Variation in plant water status has the potential to enhance anthocyanin production when the plants are under light stress. Drought can be initiated in various ways. Water can be withheld, osmolytic compounds can be applied to the soil, or plants can be treated to decrease transpiration. Water can be physically withheld prior to or during treatment, or a soil can be chosen based on its water holding capacity. Sandy soils would help promote drought, while soils high in organic matter or fine soil particles would decrease the potential for drought. Osmolytic compounds include any compound that effectively reduces water potential; therefore, applying osmolytes to the soil would prevent water from efficiently being taken up by the plant. Water potential is made up of various components, and in this instance, solute potential (Ψ_s) is the major factor. Any solute will decrease the solute potential leading to lower water potentials.

[00108] Again, lower soil water potentials initiate drought stress and serve to enhance light induced anthocyanin production. Molecules that could as sources of osmolytes include: soluble carbohydrates (for example, sucrose), salts (for example, sodium chloride, potassium chloride, salt based fertilizer, etc.), bi-carbonates, proteins, organic molecules, or any water soluble substance. Stomatal transpiration can also be hindered by the application of abscisic acid (ABA) in various concentrations to tailor drought stress. Cuticular transpiration and stomatal transpiration can also be decreased by the application of film-forming anti-transpirant compounds. These include synthetic latex, acrylic polymers, resin or pitch, and silicone emulsions. Many pesticides also contain these film forming compounds, so essentially they could also be used to efficiently cause drought stress if used at high rates.

[00109] *Nutrient stress*

[00110] Plant nutrient status must be maintained to produce adequate biomass to obtain efficient anthocyanin yield; however, nutrient depletion can also promote anthocyanin production. Specific nutrients can be withheld, applied at "toxic" levels, total fertility can be decreased, or soil pH can be adjusted. Phosphorus deficiency is noted to display purpling; withholding phosphorus prior to elicitation can serve to enhance production. Heavy metals that are either essential or non-essential plant nutrients can stimulate anthocyanin production when found in excess or toxic concentrations. Some heavy metals that could enhance anthocyanin induction include: cadmium (Cd), cobalt (Co), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn), Lead (Pb), iron (Fe), chromium (Cr), selenium (Se). Applications of these metals in many species would also interfere

with overall physiology; therefore resulting in an overall decrease in anthocyanin yield for those plants, even though anthocyanins are being produced.

[00111] Decreasing total fertility by applying less total fertilizer can also lead to enhanced anthocyanin production by making the plant more susceptible to light stress. Applying fertilizer at a lower rates (0.5 to 0.25 of the normal rate), at longer intervals, or withholding fertilizer approximately prior to elicitation would be efficient ways to decrease total fertility. The choice of soil would also affect total fertility. Sandy soils would decrease overall fertility, while soils high in organic matter or fine soil particles would increase the overall fertility. Anthocyanins have been shown to accumulate at higher levels when soil pH is towards the extremes. These conditions have direct correlations with nutrient availability and toxicity; therefore, pH can affect available phosphorus and eventually anthocyanin concentration. Maintaining an abnormally low or high pH (≤ 5 or ≥ 8) can result in enhanced anthocyanin production.

[00112] *Chemical applications*

[00113] Anthocyanins are known to accumulate due to the application of various chemicals. Both natural hormones (plant growth regulators –PGR's) as well as synthetic pesticides can enhance anthocyanin production; however, some may also decrease production. Auxins, cytokinins, ethylene, gibberellins, abscisic acid, and jasmonic acid may enhance anthocyanin production by direct regulation of PAL. Many herbicides are derivatives or mimics of plant hormones, and many times their application will result in anthocyanin induction prior to plant death. Applying low rates of natural hormones or various synthetic derivatives has the potential to further enhance production. Any herbicide that alters phenylpropanoid metabolism would have the potential to increase anthocyanin concentration; therefore applying one of these chemicals prior to or during elicitation may enhance anthocyanin accumulation.

[00114] *Species that could be used for vegetative (shoot tissues) anthocyanin elicitation*

The following examples of species represent a non-exhaustive list of plants having one or more of the following qualities: plants that A) produce valuable anthocyanins, B) Possess the anatomy/physiology to allow production using the same plant, and/or C) Annual plants that are known for the ability to induce anthocyanins.

Table 9: C3 turfgrass species	
Kentucky Bluegrass	<i>Poa pratensis</i>
Perennial Rye	<i>Lolium perenne</i>
Tall Fescue	<i>Schedonorus phoenix</i>
Hard Fescue	<i>Festuca brevipila</i>
Chewings Fescue	<i>Festuca rubra</i> ssp. <i>commutata</i>
Creeping Bentgrass	<i>Agrostis stolonifera</i>
Colonial Bentgrass	<i>Agrostis capillaris</i>
Strong Creeping Red Fescue	<i>Festuca rubra</i> ssp. <i>rubra</i>
Slender Creeping	<i>Festuca rubra</i> ssp.

Red Fescue	<i>litoralis</i>
Velvet Bentgrass	<i>Agrostis canina</i>
Rough Bluegrass	<i>Poa trivialis</i>
Annual Bluegrass	<i>Poa annua</i>
Annual Ryegrass	<i>Lolium multiflorum</i>
Canadian bluegrass	<i>Poa compressa</i>
Supine bluegrass	<i>Poa supina</i>
Weak meadowgrass	<i>Poa infirma</i>
Alpine meadowgrass	<i>Poa alpina</i>

Table 10: C4 turfgrass species	
Japanese lawngrass	<i>Zoysia japonica</i>

Manilla grass	<i>Zoysia matrella</i>
Hybrid Bermudagrass	<i>Cynodon dactylon</i> x <i>Cynodon transvalensis</i>
Common Bermudagrass	<i>Cynodon dactylon</i>
Centipedegrass	<i>Eremochloa ophiuroides</i>
Seashore Paspalum	<i>Paspalum vaginatum</i>
Buffalograss	<i>Buchloe dactyloides</i>
St. Augustinegrass	<i>Stenotaphrum secundatum</i>
Kikuyugrass	<i>Pennisetum clandestinum</i>
Bahiagrass	<i>Paspalum notatum</i>

Table 11: Other monocots

Corn (Maize)	<i>Zea mays</i>
Sorghum	<i>Sorghum bicolor</i>
Johnson grass	<i>Sorghum halepense</i>
Orchard grass	<i>Dactylis glomerata</i>
Giant crabgrass	<i>Digitaria sanguinalis</i>
Smooth crabgrass	<i>Digitaria ischaemum</i>
Giant foxtail	<i>Setaria faberi</i>
Green foxtail	<i>Setaria viridis</i>
Yellow foxtail	<i>Setaria glauca</i>
Redtop bentgrass	<i>Agrostis gigantea</i>
Oat	<i>Avena sativa</i>
Wheat	<i>Triticum spp</i>
Rye	<i>Secale cereale</i>
Barnyard grass	<i>Echinochloa crus-galli</i>
Switchgrass	<i>Panicum virgatum</i>
Fall panicum	<i>Panicum dichotomiflorum</i>
Asian rice	<i>Oryza sativa</i>

Black rice	<i>Oryza sativa L. indica</i>
Rice spp.	<i>Oryza spp.</i>
Barley	<i>Hordeum vulgare</i>
Phyllostachys (bamboo spp.)	<i>Phyllostachys spp.</i>
Timothy grass	<i>Phleum pratense</i>

Table 12: Dicots

Red cabbage	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>rubra</i>
White clover	<i>Trifolium repens</i>
Red clover	<i>Trifolium pratense</i>
Alfalfa	<i>Medicago sativa</i>
Creeping woodsorrel	<i>Oxalis corniculata</i>
Yellow woodsorrel	<i>Oxalis stricta</i>
Virginia creeper	<i>Parthenocissus quinquefolia</i>
Red root pigweed	<i>Amaranthus retroflexus</i>
Red amaranth	<i>Amaranthus cruentus</i>
Prostrate spurge	<i>Euphorbia supina</i>
Prostrate pigweed	<i>Amaranthus blitoides</i>
Henbit	<i>Lamium amplexicaule</i>
Ground ivy	<i>Glechoma hederacea</i>
Common mallow	<i>Malva neglecta</i>
Arabadopsis	<i>Arabadopsis thaliana</i>
Chickweed	<i>Stellaria media</i>
Mouseear chickweed	<i>Cerastium vulgatum</i>

[00115] Example 6: Elicitation stress combination with UV and osmotic stress

[00116] White clover was seeded into a soil consisting of 80% sand, 10% soil, and 10% compost, and was allowed to grow for 3 months prior to elicitation. 1 week prior to stress induction, 0.10 lb. nitrogen/1000 ft² was applied to all plants to maintain low fertility prior to elicitation. White clover (*Trifolium repens*) was then treated with a low rate of jasmonic acid (10 PPM) 1 day prior to elicitation. Elicitation was implemented using a high wattage blue LED array producing 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ on average. UV-A and UV-B LED's were also supplemented on the LED array to produce a total UV irradiance of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. To induce an initial state of drought, plants were watered with a 10% sucrose solution, and were not watered for the following 2 days. A "day" temperature was set to fluctuate between 10-18°C every 30 minutes for 12 hours,

and a “night” temperature was set to 5°C. Lights were allowed to run for 24 hours, and plants were harvested following 5 days of elicitation.

[00117] *Example 7: Elicitation stress combination with very high light, UV, ethylene, and temperature fluctuation*

[00118] Bermudagrass (*Cynodon dactylon*) was sprigged into a 100% sand soil, was fertilized weekly at a rate of 1 lb. nitrogen/1000 ft² weekly, and was allowed to grow for approximately 3 months prior to elicitation. Two weeks prior to elicitation all fertilizer applications were withheld, and two days before elicitation plants were treated with ABA to initiate a state of drought stress. Elicitation was implemented using a high wattage white LED array producing 2,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ on average, and UV-A and UV-B LED's were also supplemented on the LED array to produce a total UV irradiance of 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$. During elicitation, ethylene vapor (250 PPM) was released into the chamber for 10 minutes every other day to further enhance anthocyanin production. Plants were exposed to temperature fluctuation between 0-10 °C every hour, and lights were set to run for 16 hours. Following 10 days of elicitation, plant tissue was harvested.

[00119] The use of a variety of stressors may be used to enhance anthocyanin production in treated plants as compared with plants grown under standard growing conditions.

[00120] *Standard growing conditions*

[00121] Plants produced by the methods described may be compared with plants grown under standard growing conditions. Standard growing conditions include growth in nutrient-rich soil, under diurnal light and temperature fluctuation, and provided with adequate water. Temperatures for plants are generally maintained within a range of approximately 15-25°C, not exceeding 10-30°C. Daylight periods generally do not exceed 16hrs/day. Standard growing conditions may include the application of fertilizers and growth in outdoor fields or indoor greenhouses. Standard conditions may vary depending on the plant variety cultivated. Plants grown under standard growing conditions do not exhibit stress symptoms.

[00122] *Plant stress measurement*

[00123] Plant stress may be measured by a variety of methods. For example: chlorophyll fluorometers, chlorophyll content meters, quenching measurements photosynthesis systems, such as infrared gas analyzers, Fv/Fm, Y(II), electron transport rate, and combinations of these methods.

[00124] For example, Fv/Fm tests whether or not plant stress affects photosystem II in a dark adapted state. Light that is absorbed by a leaf follows three competitive pathways. It may be used in photochemistry to produce ATP and NADPH used in photosynthesis, it can be re-emitted as fluorescence, or dissipated as heat. The Fv/Fm test is designed to allow the maximum amount of the light energy to take the fluorescence pathway. It compares the dark-adapted leaf pre-photosynthetic fluorescent state, called minimum fluorescence, or F₀, to maximum fluorescence, or Fm. In maximum fluorescence, the maximum number of reaction centers have been reduced or

closed by a saturating light source. In general, the greater the plant stress, the fewer open reaction centers available, and the Fv/Fm ratio is lowered. Fv/Fm is a measuring protocol that works for many types of plant stress.

[00125] *Example 8: Detailed Cultivar Comparison*

[00126] Evaluation was made of ecotypic, accessional variability in rough bluegrass *Poa trivialis* (RB). Understanding of morphological and physiological variability provides critical data for optimization. Assessment was made of 25 accessions, or cultivars, of RB from across the world. RB is frequently a nuisance plant in cultivated lawns where its color and appearance may differ from lawn grasses such as Kentucky bluegrass or perennial ryegrass. It has a low stress tolerance for environmental conditions such as heat and drought.

[00127] **Table 13** identifies cultivars and anthocyanin content in the assessed plants.

Measurement is by mg/100mg of freeze dried leaf tissue.

[00128] **Table 13: Sampled Anthocyanin Content of Cultivars Analyzed**

Acc. I.D.	mg/100g freeze dried tissue	Accession or Cultivar		Origin
1	9.43	PI 221908	HIJ	Afghanistan
2	5.26	PI 221915	J	Afghanistan
3	14.07	PI 221951	EFGHI	Afghanistan
4	18.08	PI 659652	DEFG	Czech Republic
5	10.76	PI 225826	GHIJ	Denmark
6	21.21	W6 2820	DEF	Germany
7	31.45	W6 28122	BC	Germany
8	79.42	W6 28268	A	Germany
9	23.39	PI 227672	CD	Iran
10	16.73	PI 227858	DEFGH	Iran
11	11.2	PI 229719	GHIJ	Iran
12	14.43	PI 251417	EFGHI	Iran
13	11.78	PI 380993	GHIJ	Iran
14	5.38	PI 254908	J	Iraq
15	17.43	PI 659892	DEFGH	Kyrgyzstan
16	10.6	PI 422592	GHIJ	Morocco
17	12.54	PI 578852 'PO-LIS'	GHIJ	Netherlands
18	12.97	PI 250982	EFGHI	Serbia/Montenegro
19	12.1	PI 251167	GHIJ	Serbia/Montenegro
20	8.17	PI 204484	IJ	Turkey
21	32.87	PI 537439 'LASER'	B	USA
22	23.79	PI 592521 'ProAm'	CD	USA
23	21.6	PI 594396 'SABRE'	DE	USA
24	21.44	PI 601315 'COLT'	DE	USA
25	21.81	'Havana'	DE	USA

[00129] The response to elicitation can vary widely. In selecting a plant for anthocyanin cultivation, the relative value of constituent production can be dwarfed by elicitation responsiveness. For example, the Havana cultivar of *Poa trivialis* L. exhibited a constituent anthocyanin concentration of 1.6mg/100g and a stressed induced concentration of 186mg/100g, or

an increase of approximately 11,500% after elicitation.

[00130] The morphology and physiology of ten accessions were assessed in greater detail. The plants were cut and material harvested from all tissue 1.5 cm from the top of the pot. Acetone extraction and chloroform separation were performed on the plant tissue. The pH differential method was used to determine anthocyanin concentration.

[00131] The total cuticular wax in relation to anthocyanin production was evaluated (**FIG. 18A, FIG. 18B**). Wax was negatively correlated with anthocyanin. Increasing wax also decreases sensitivity to blue light as a trigger for anthocyanin production. Total cuticular wax was assessed by chloroform extraction and quantified by the potassium dichromate method.

[00132] Mesophyll thickness was assessed relative to anthocyanin concentration (**FIG. 17A, FIG. 17B, and FIG. 17C**). In general, varieties showing thinner mesophyll produced more anthocyanin. Samples of leaf tissue were assessed by Scanning Electron Microscopy (SEM) after Critical Point Drying (CPD) preservation. Samples were also assessed by standard microscopy.

[00133] The precursor effects of phenolics and flavonoids were evaluated (**FIG. 19A, FIG. 19B**). Total phenolic and flavonoid content were assessed prior to and 48 hours into treatment. Phenolics were evaluated by Folin's phenol reagent method. Flavonoids were evaluated by the aluminum chloride method.

[00134] *Example 9: Use of anthocyanin applications to mitigate plant stress*

[00135] High light level and high UV are examples of plant stressors that can be effectively treated with topical application of anthocyanins to foliage. In experimental studies, anthocyanin application was as effective as pigment green 7 (phthalocyanine), at reducing light stress in turfgrass.

[00136] All pigments, natural or synthetic, have the ability to selectively absorb, transmit, and reflect various wavelengths of light. To help mitigate light stress, UV or visible, plants accumulate screening compounds like anthocyanins. By extracting anthocyanins and combining them with a given adjuvant system, the anthocyanins can be sprayed on plant foliage to help reflect and absorb potentially damaging light while still allowing the transmission of photosynthetically active radiation (**FIG. 25**).

[00137] To determine if synthetic pigments have the ability to decrease plant stress the following experiment was performed. Treatments were applied consisting of an untreated control, 500 PPM green 7 solution, 1,000 PPM green 7, and 2,000 PPM green 7. Lab grade pigment green 7 was obtained through Fisher Scientific (Alfa Aesar) and was used to make a high concentration stock solution. Green 7 was dispersed into solution using TritonTM X-100 surfactant and heat. Once evenly dispersed, this stock solution was used to make solutions of 500, 1,000, and 2,000 PPM green 7.

[00138] Plots of 0.9 x 1.8 m were setup at The Ohio Turfgrass Foundation Research and

Education Facility (2710 North Star Rd., Columbus, OH 43210) in a randomized complete block design consisting of 3 blocks. Species population consisted of approximately 80% creeping bentgrass (*Agrostis stolonifera*) and 20% annual bluegrass (*Poa annua*), root zone construction followed USGA specifications, and mowing took place 7 days / week at a height of .4318 cm. Treatments were sprayed with a CO₂ powered sprayer at 40 psi. A boom with 2 Teejet 6503 flat fan nozzles (65° spray angle & .30 gallons/minute) spaced at 50.8cm was used to apply all treatments with an output of 7.57 L/M at a walking speed of 1.5'/second.

[00139] To determine the effects of applications of pigment green 7, varying concentrations of the pigment were applied to a creeping bentgrass putting green-type lawn. During mid-day hours, when light stress is at its peak, a 2,000 PPM green 7 solutions was shown to increase photochemical efficiency compared to an untreated control. These results show a dose dependent effect, with high concentrations of green 7 showing a positive effect on bentgrass physiology.

[00140] Data was taken on photochemical efficiency (Fv/Fm) approximately at noon for each collection period. Photochemical efficiency was measured using an Opti-Sciences chlorophyll fluorometer model OS-30p. Opti-Sciences dark adaption leaf clips designed for this fluorometer were divided in half, and the top half was used for measurement (cuvette only). In the field, the cuvette was placed on the turfgrass canopy and was stapled in place using paper clips. 4 sub-samples per plot were determined to be sufficient following testing for the precision of this method. Measurements were taken one block at a time, and all samples were dark adapted for 10-15 minutes prior to analysis. Statistics were performed using SAS 9.2 and the MIXED procedure. Results show a dose dependent effect (**FIG. 21** and **Table 14**). Examining the 95% confidence interval, treatments are clearly separated by concentration. Only the high rate, 2,000 PPM, was significantly different than the untreated control. Therefore, high rates of green 7 have the potential of mitigating light stress.

[00141] **Table 14:** Comparison by concentration

Table 14			
Treatment	Lower bound (Fv/Fm)	LS mean (Fv/Fm)	Upper bound (Fv/Fm)
UTC	0.722	0.729	0.735
500 PPM	0.727	0.734	0.741
1,000 PPM	0.734	0.740	0.747
3,000 PPM	0.742	0.749	0.756

Lower bound = LS mean – SE

Upper bound = LS mean + SE

SE = .007

[00142] Rather than using synthetic compounds, anthocyanins can be used in the same fashion as pigment green 7 for mitigating light stress. Rough bluegrass tissue rich in anthocyanins that had been previously freeze dried was ground in liquid nitrogen and extracted in acetone. The extract was subjected to C-18 column chromatography, where once bound to the column anthocyanins

were washed with one volume of distilled water and eluted using a volume of .1% acidified (HCL) methanol. This washing step was beneficial to remove soluble sugars that may lead to pathogen infection or insect damage while still leaving other phenolics and flavonoids in solution. The resulting methanolic extract was then evaporated under reduced pressure to concentrate anthocyanins and remove the alcohol. A small volume (5-10 mL) of distilled water was added to the concentrated extract, and the concentration of the solution was determined by the pH differential method.

[00143] For spraying, a 400 PPM anthocyanin solution was made using the previous concentrated extract, pH was adjusted to 3.0 using concentrated HCL or NaOH, and Bond® Spreader Sticker was added at a rate of .8 mL/L to aid in leaf deposition. Solutions were sprayed onto previously sown pots of creeping bentgrass using a small volume hand pumped sprayer and allowed to dry for 24 hours prior to treatment. For comparison, pigment green 7 was sprayed on separate creeping bentgrass plants at a concentration of 1,000 PPM (16 oz./Acre). Stress treatments were applied over a 27 hr. period where plants were exposed to continuous white light using metal halide HID lamps. The growth chamber was set to a constant temperature of 18°C, and light intensity was adjusted over time from 600-1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. All treatments were made using 3 replicates and were blocked by placement under individual metal halide lamps.

[00144] Data was taken on photochemical efficiency (Fv/Fm), chlorophyll content, and chlorophyll *a:b* ratio. Photochemical efficiency was measured using an Opti-Sciences chlorophyll fluorometer model OS-30p. Opti-Sciences dark adaption leaf clips designed for this fluorometer were divided in half, and the top half was used for measurement (cuvette only). Three Fv/Fm sub-samples per pot were measured throughout the 27 hr. period, and all samples were dark adapted for 10-15 minutes prior to analysis. Chlorophyll was extracted using N,N-dimethylformamide and chlorophyll *a* and *b* concentrations were spectrophotometrically determined.

[00145] Following light treatment, results showed that both chlorophyll parameters, total content and *a:b* ratio, responded in the same manner for all three treatments (**FIGS. 22, 23, 24 and Table 15**). Data shows that there were no negative physiological effects of the anthocyanin spray at 400 PPM. Possible negative effects could have been associated with high levels of shading or any associated phytotoxicity.

[00146] **Table 15:** Total chlorophyll content and chlorophyll *a:b* ratio

Table 15				
Treatment	Total chlorophyll (mg/g)		Chlorophyll <i>a:b</i> ratio	
Pre-treatment	3570.9	A	3.04	A
UTC	2922.5	B	2.93	B
Anthocyanin Spray	2952.3	B	2.89	B
Green 7	3028.5	B	2.96	B

LSD

284.49

0.079

[00147] A sticker can be added to increase the adhesion or "stickiness" of solid particles, like powdered anthocyanin compounds, that otherwise might be easily dislodged from a leaf surface and also to provide a waterproof coating. Many of the stickers contain surfactants as their principal functioning agent and function as spreader-stickers, which give both a sticker action and a wetter-spreader action. But the surfactants, providing wetter-spreader action must be somewhat water soluble, and so may not provide good protection from rain. This will be provided by products that contain latex (rubber), polyethylene (plastic), resins (rosin), polymethenes (rosin-like), or other waterproofing agents. For example, a good sticker for use with anthocyanin adhesion to foliage would include Bond® Spreader Sticker Deposition Aid (Loveland Products), comprised of synthetic latex 1,2-propanediol, alcohol ethoxylate. Other examples include: Tactic™ Sticker – Organosilicone surfactant – deposition agent (Loveland Products), R-56® Spreader Sticker (Wilbur-Ellis Co.), and Cohere® Nonionic Spreader Sticker (Helena Chemical Co.).

[00148] In one embodiment, the treatment comprises an anthocyanin, at a concentration of 400 ppm, with Bond®, at a concentration of 0.8mL/L, adjusted to approximately pH 3.0, and applied at approximately 0.25-10 L/acre. In some embodiments, anthocyanins can reduce the photodegradation of pesticides. Dyes and pigments could be added to adjust the color and visual appeal.

[00149] Compositions according to the instant invention generally contain from about 0.5 to about 95% of anthocyanin by weight, preferably from 1% to 50%, more preferably from 2% to 35%. The remainder of the composition comprises a carrier as well as various optional additives such as those described herein.

[00150] "Carrier" is meant herein as an organic or inorganic material, which can be natural or synthetic and is associated with the anthocyanin and facilitates its application to the plants to be treated. This carrier is thus generally inert and should be agriculturally acceptable. The carrier can be solid (e.g., clay, silicates, silica, resins, wax, fertilizers, or the like) or liquid (e.g., water, alcohols, ketones, oil solvents, saturated or unsaturated hydrocarbons, chlorinated hydrocarbons, liquefied petroleum gas, or the like).

[00151] Among the many optional additives suitable for use in compositions with anthocyanins include surfactants and other ingredients, such as dispersants, stickers, antifoam agents, antifreezing agents, dyes, pigments, thickeners, adhesives, protective colloids, penetrating agents, stabilizing agents, sequestering agents, antiflocculating agents, corrosion inhibitors, pesticides, fungicides, and polymers. More generally, the compositions of the invention can include all kinds of solid or liquid additives which are known in the art of crop protection and horticultural treatments.

[00152] The surfactants can be of the emulsifying or wetting type and can be ionic or non-ionic. Possible surfactants are salts of polyacrylic or lignosulfonic acids; salts of phenolsulfonic or naphthalenesulfonic acids; polycondensates of ethylene oxide with fatty alcohols or fatty acids or fatty amines or substituted phenols (particularly alkylphenols or arylphenols); ester-salts of sulfosuccinic acids; taurine derivatives, such as alkyl taurates; phosphoric esters; or esters of alcohols or polyoxyethylated phenols.

[00153] Dusting powders, granulates, solution, emulsifiable concentrates, emulsions, suspended concentrates and aerosols are also contemplated. The wettable powders according to the invention can be prepared in such a way that they contain from 1% to 95% by weight of the active material, and they normally contain, in addition to a solid support, from 0 to 5% by weight of a wetting agent, from 3 to 10% by weight of a dispersant, and, when necessary, from 0 to 10% by weight of one or more stabilizers and/or other additives, such as penetration agents, adhesives or anti-clumping agents, or colorants. The compositions can contain other ingredients, for example protective colloids, adhesives or thickeners, thixotropic agents, stabilizers or sequestrants, as well as other active materials known to have pesticidal properties, especially certain fungicides, acaricides, and insecticides.

[00154] The method can be practiced on grasses, including those used for lawns or other ornamental purposes, such as turfgrass, and those used as food or to produce grain for human or animal consumption. Some grasses, such as rye grasses, can be used both for food and for esthetic purposes. Turfgrasses are typically characterized as cool season turfgrasses and warm season turfgrasses. Examples of cool season turfgrasses are bluegrasses (*Poa* spp.), such as Kentucky bluegrass (*Poa pratensis* L.), rough bluegrass (*Poa trivialis* L.), Canada bluegrass (*Poa compressa* L.), annual bluegrass (*Poa annua* L.), upland bluegrass (*Poa glaucantha* Gaudin), wood bluegrass (*Poa nemoralis* L.), and bulbous bluegrass (*Poa bulbosa* L.); the bentgrasses and redtop (*Agrostis* spp.), such as creeping bentgrass (*Agrostis palustris* Huds.), colonial bentgrass (*Agrostis tenuis* Sibth.), velvet bentgrass (*Agrostis canina* L.), South German Mixed Bentgrass (*Agrostis* spp. including *Agrostis tenuis* Sibth., *Agrostis canina* L., and *Agrostis palustris* Huds.), and redtop (*Agrostis alba* L.); the fescues (*Festuca* spp.), such as red fescue (*Festuca rubra* L. spp. *rubra*), creeping fescue (*Festuca rubra* L.), chewings fescue (*Festuca rubra commutata* Gaud.), sheep fescue (*Festuca ovina* L.), hard fescue (*Festuca longifolia* Thuill.), hair fescue (*Festuca capillata* Lam.), tall fescue (*Festuca arundinacea* Schreb.), meadow fescue (*Festuca elanor* L.); the ryegrasses (*Lolium* spp.), such as annual ryegrass (*Lolium multiflorum* Lam.), perennial ryegrass (*Lolium perenne* L.), italian ryegrass (*Lolium multiflorum* Lam.); and the wheatgrasses (*Agropyron* spp.), such as fairway wheatgrass (*Agropyron cristatum* (L.) Gaertn.), crested wheatgrass (*Agropyron desertorum* (Fisch.) Schult.), and western wheatgrass (*Agropyron smithii* Rydb.). Other cool season turfgrasses include beachgrass (*Ammophila breviligulata* Fern.),

smooth brome grass (*Bromus inermis* Leyss.), cattails such as Timothy (*Phleum pratense* L.), sand cattail (*Phleum subulatum* L.), orchardgrass (*Dactylis glomerata* L.), weeping alkaligrass (*Puccinellia distans* (L.) Parl.) and crested dog's-tail (*Cynosurus cristatus* L.). Examples of warm season turfgrasses include Bermudagrass (*Cynodon* spp. L. C. Rich), zoysiagrass (*Zoysia* spp. Willd.), St. Augustine grass (*Stenotaphrum secundatum* Walt Kuntze), centipedegrass (*Eremochloa ophiuroides* Munro Hack.), carpetgrass (*Axonopus affinis* Chase), Bahia grass (*Paspalum notatum* Flugge), Kikuyugrass (*Pennisetum clandestinum* Hochst. ex Chiov.), buffalo grass (*Buchloe dactyloids* (Nutt.) Engelm.), Blue gramma (*Bouteloua gracilis* (H.B.K.) Lag. ex Griffiths), seashore paspalum (*Paspalum vaginatum* Swartz) and sideoats grama (*Bouteloua curtipendula* (Michx. Torr.). Cool season turfgrasses may benefit preferentially from treatment and protection from light or heat stress. Examples of grasses that are useful as crops include corn or maize (*Zea mays*), sorghum (*Sorghum sudanense*), switchgrass (*Panicum virgatum*), millet (*Panicum miliaceum*), rice (*Oryza* spp.), wheat (*Triticum* spp.), oats (*Avena* spp.), barley (*Hordeum* spp.), and rye (*Secale cereale*).

[00155] In general the rate of application is from 0.001 to 10 kilograms of anthocyanin per hectare (kg/ha), from about 0.01 to about 2 kg/ha, from about 0.1 to about 1 kg/ha, and from about 0.2 to about 0.8 kg/ha.

[00156] Citation of any of the documents recited herein is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00157] While the invention has been described with reference to various and preferred embodiments, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the essential scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope thereof.

[00158] Therefore, it is intended that the invention not be limited to the particular embodiment disclosed herein contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the claims.

CLAIMS

What is claimed is:

1. A method for increasing anthocyanin production in a plant comprising:
 - a) selecting a plant;
 - b) cultivating the plant;
 - c) applying stress to the plant, wherein the stress comprises: subjecting the plant to light stress comprising: intense synthetic light and long duration light exposure;
 - d) harvesting plant tissue; and
 - e) repeating steps b, c, and d.
2. A method for eliciting anthocyanin production in a plant comprising:
applying light to the plant, wherein the light color is substantially white or blue, wherein the light intensity is at least $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, for at least 100 continuous hours, and wherein the elicitation produces at least 50mg of anthocyanins per 100g foliage.
3. A method of plant treatment for the elicitation of anthocyanin production in a plant, wherein a majority of the above-ground plant tissue is harvested at least once in a single growing season, the treatment comprising: cultivating the plant; applying stress, wherein the stress comprises: subjecting the plant to light stress comprising: intense light and long duration light exposure; harvesting the majority of the above-ground plant tissue; and extracting anthocyanins from the tissue.
4. The method of claim 3, wherein the yield of anthocyanin is increased by 50-200% as compared with a control plant, of the same variety, grown under standard conditions.
5. The method of claim 3, wherein the light stress further comprises continuous exposure to blue light, at an intensity of $400\text{-}1500 \mu\text{mol m}^{-2} \text{s}^{-1}$, for four to seven days.
6. The method of claim 3, wherein the plant is red cabbage.
7. A plant produced by the method of claim 3.
8. The method of claim 1, wherein steps b, c, and d are performed sequentially at least twice.
9. The method of claim 1, wherein steps b, c, and d are performed sequentially at least three times.
10. The method of claim 1, wherein the plant selected is a turfgrass.

11. The method of claim 1, wherein the plant is *Poa trivialis*.
12. The method of claim 1, wherein the plant is *Agrostis stolonifera*.
13. The method of claim 1, wherein the plant is selected from the group consisting of the plants listed in Table 9, Table 10, Table 11, and Table 12.
14. The method of claim 1, wherein the plant selected is not transgenic.
15. The method of claim 1, wherein the stress further comprises osmotic stress.
16. The method of claim 1, wherein the stress further comprises osmotic stress, wherein water is not applied for a period of greater than 24 hours.
17. The method of claim 1, wherein the stress further comprises osmotic stress, wherein the osmotic stress is selected from the group consisting of: withholding water; treatment of foliage with a film-forming anti-transpirant; application of abscisic acid; and application of one or more osmolytes, wherein the osmolyte is sugar, salt, fertilizer, bi-carbonate, protein, or a water soluble organic molecule.
18. The method of claim 1, wherein the light stress comprises a light duration of 24 hours/day for at least 3 days.
19. The method of claim 1, wherein the light stress comprises sunlight in combination with synthetic light, wherein total instantaneous photosynthetic photon flux is approximately $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$.
20. The method of claim 1, wherein the light stress comprises white light.
21. The method of claim 1, wherein the light stress comprises blue light.
22. The method of claim 1, wherein the light stress comprises blue light wherein the wavelength is approximately 450-495nm.
23. The method of claim 1, wherein the light stress comprises white light having a measured instantaneous photosynthetic photon flux of approximately $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$.
24. The method of claim 1, wherein the light stress comprises white light having a measured instantaneous photosynthetic photon flux within a range of 900-1,200 $\mu\text{mol m}^{-2} \text{s}^{-1}$.
25. The method of claim 1, wherein a light source comprises a high intensity discharge (HID) lamp.

26. The method of claim 1, wherein a light source comprises a light-emitting diode (LED).
27. The method of claim 1, wherein the stress further comprises cool temperature.
28. The method of claim 1, wherein the stress further comprises a growing temperature between 0-15°C.
29. The method of claim 1, wherein the stress further comprises a growth medium comprising 75-100% sand.
30. The method of claim 1, wherein the stress further comprises a growth medium low in phosphate.
31. The method of claim 1, wherein the majority, by weight, of harvested plant tissue comprises leaves.
32. The method of claim 1, wherein the plant selected produces less than 1% dry weight anthocyanins under standard growing conditions, wherein standard growing conditions are conditions optimal for biomass production and biomass yield, and wherein the plant produces 2-20% dry weight anthocyanins under the method of claim 1.
33. The method of claim 1, wherein greater than 50% of the anthocyanin produced is cyanidin 3 malonyl-glucoside.
34. The method of claim 1, wherein the plant yield of anthocyanin is greater than 1g/kg dry weight.
35. An ornamental plant in which a red or purple coloration is elicited by the application continuous bright light, at 400-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, for at least 30 to 100 hours at a temperature of 0-15°C, wherein the elicitation produces 20-200mg/100g fresh weight of anthocyanins in foliage and wherein the plant produces less than 15mg/100g fresh weight of anthocyanins in foliage under standard growing conditions.
36. A photoprotective composition comprised of anthocyanins, wherein the composition is adapted for application to reduce stress in plants.
37. The composition of claim 36 wherein the stress is exposure to high levels of ultraviolet radiation and/or visible light.
38. The composition of claim 36 further comprising: at least one sticker and at least one carrier.

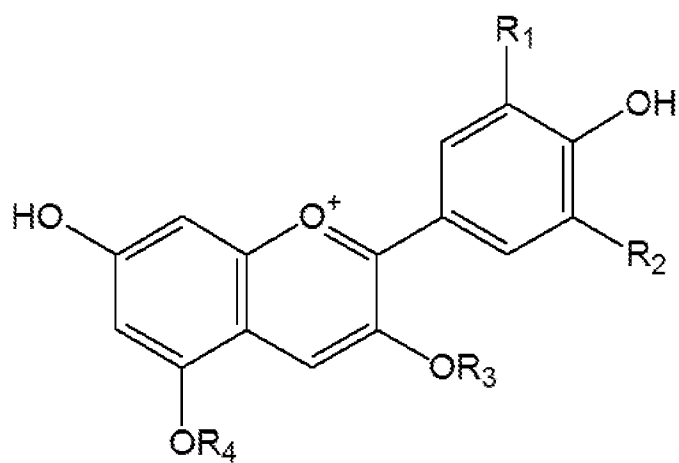


FIG. 1

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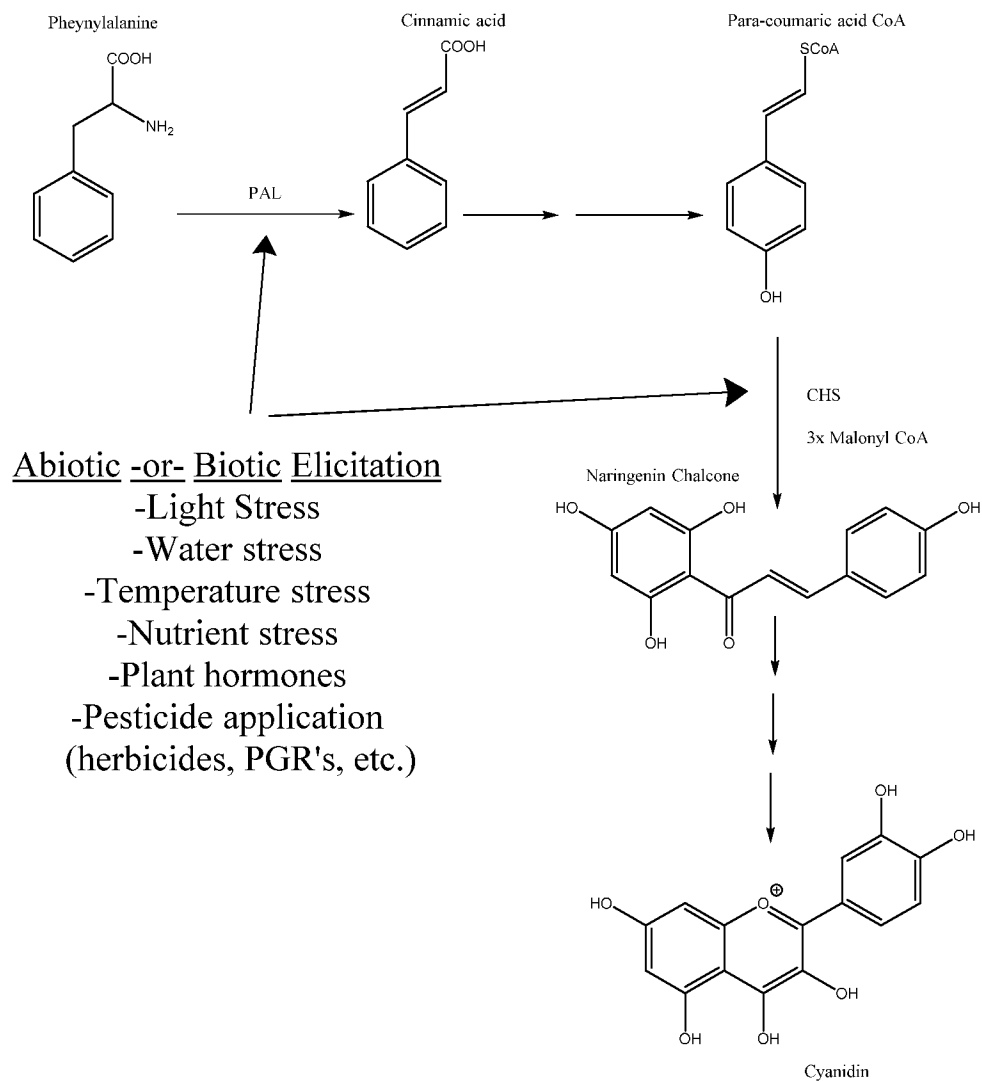
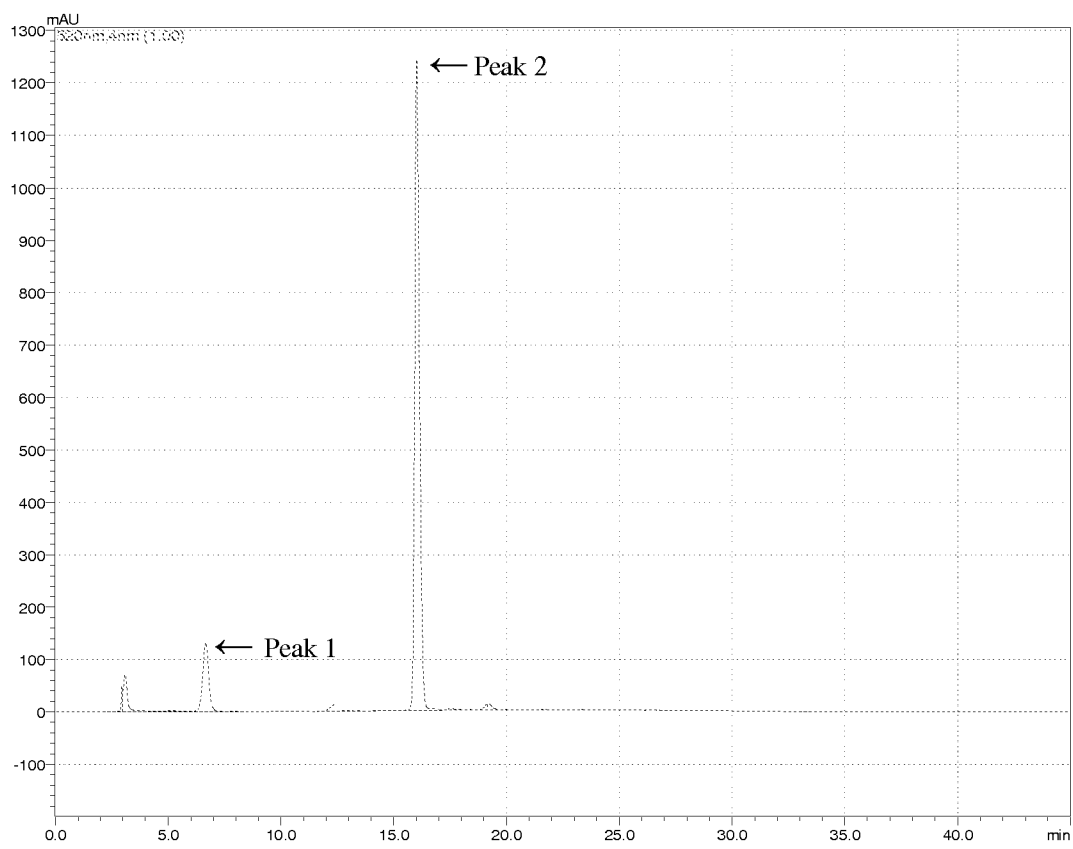


FIG. 2



Peak	T _r (min) [†]	[M] [‡] m / z [‡]	Fragments m / z [§]	UV – vis nm [¶]	Identity
1	6.88	449	287	280,516	Cyanidin 3-glucoside
2	15.61	535	287	281,519	Cyanidin 3-malonyl glycoside

[†] Retention time.
[‡] Mass of whole compound.
[§] Breakdown of compound sizes after passage through mass spectrometer.
[¶] Peak absorption of compounds in UV and PAR regions.

FIG. 3

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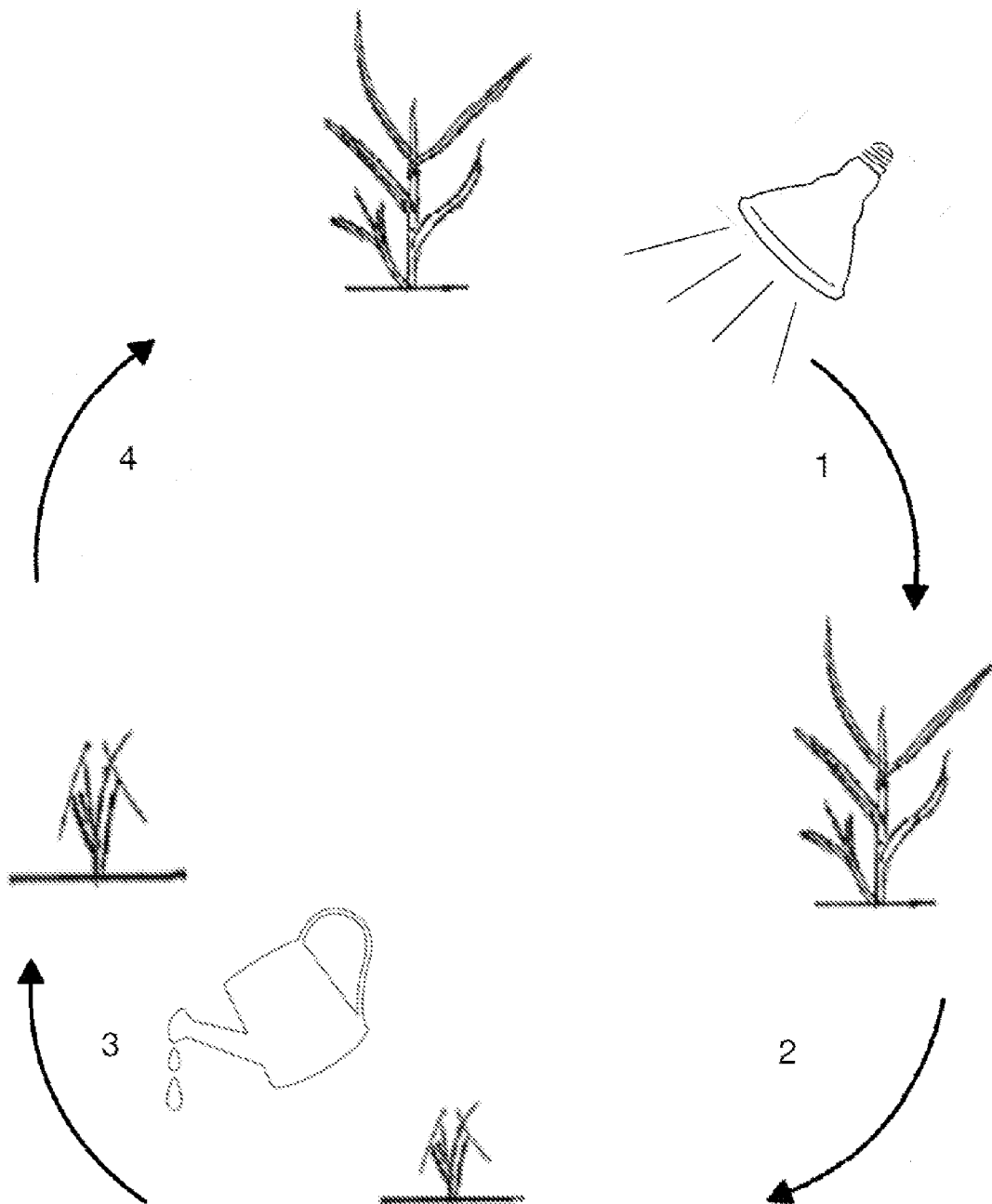


FIG. 4

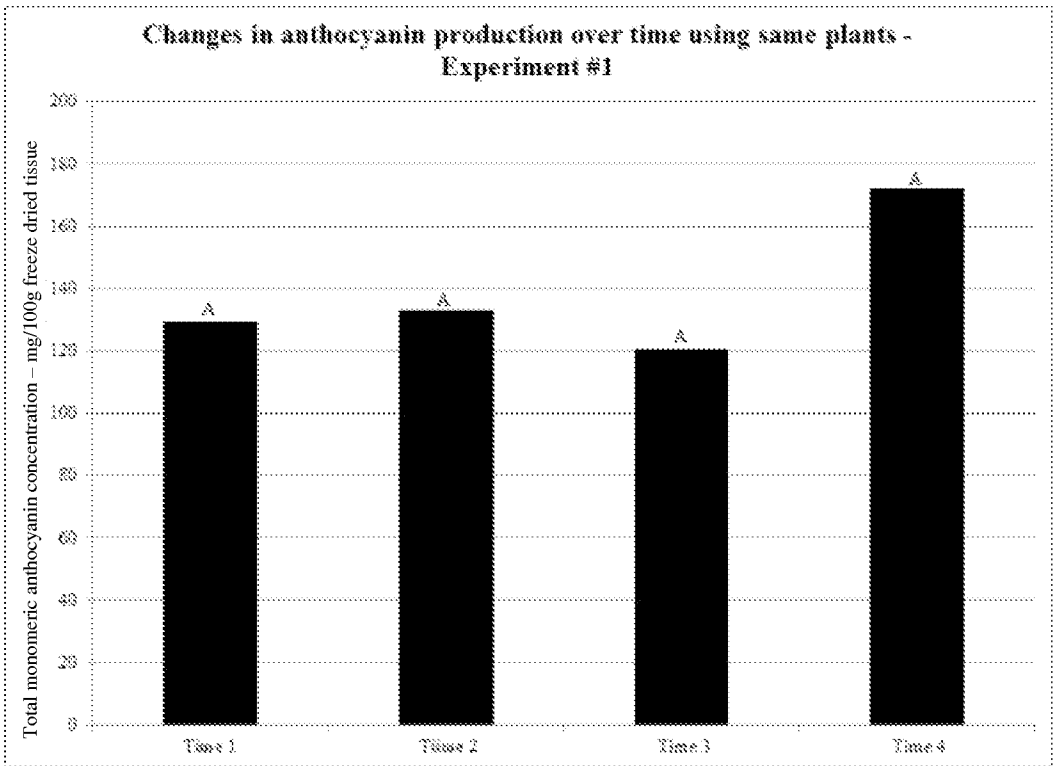


FIG. 5

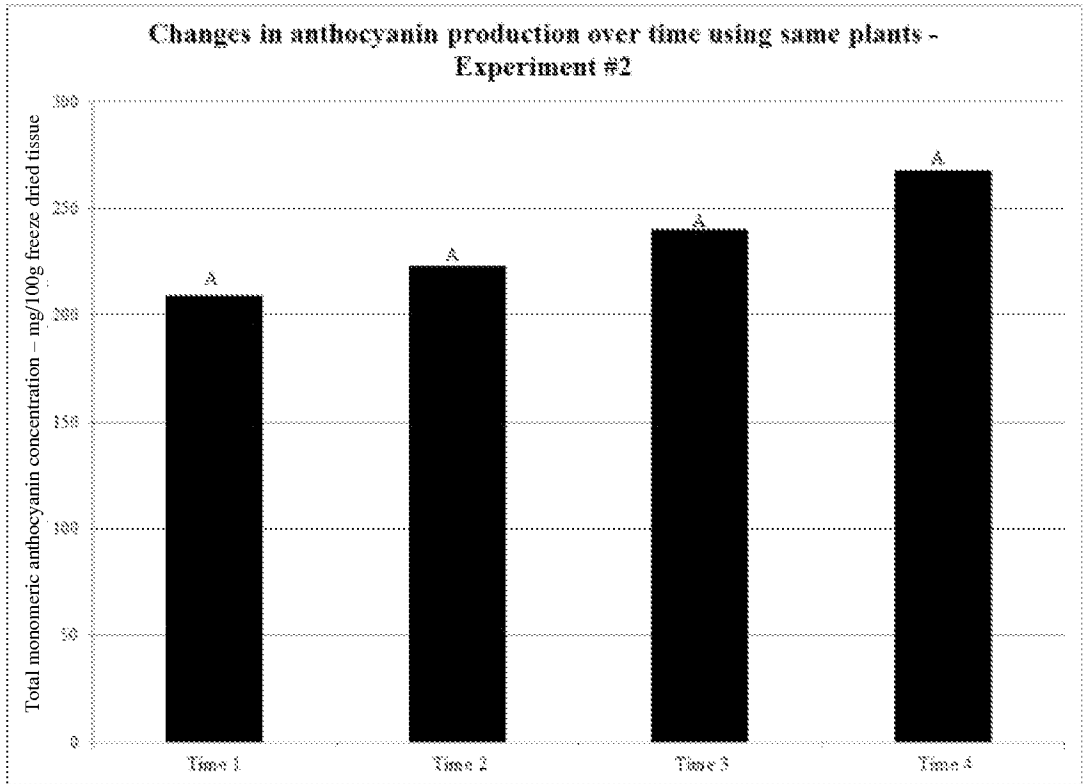


FIG. 6

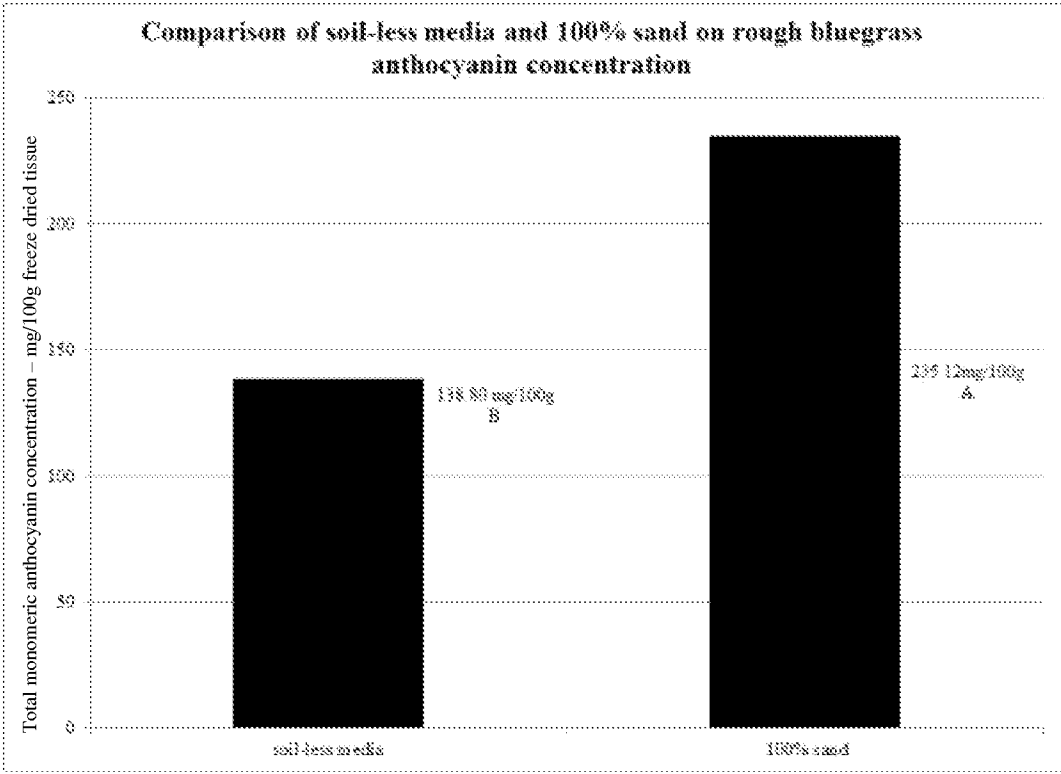


FIG. 7

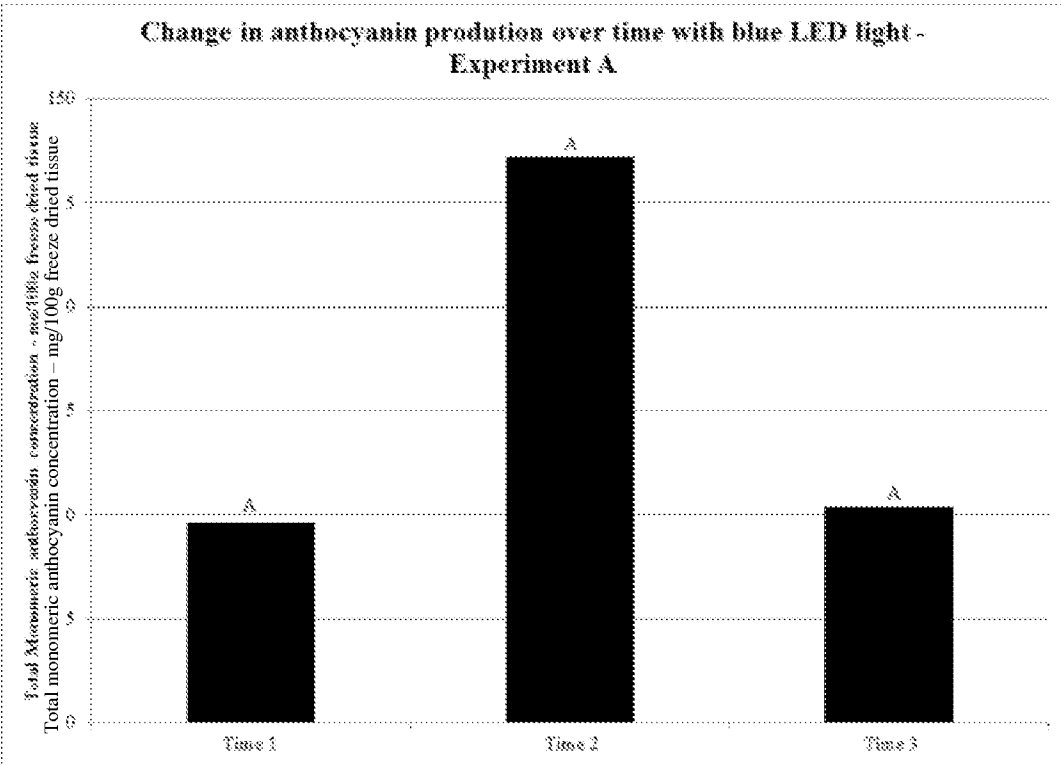


FIG. 8

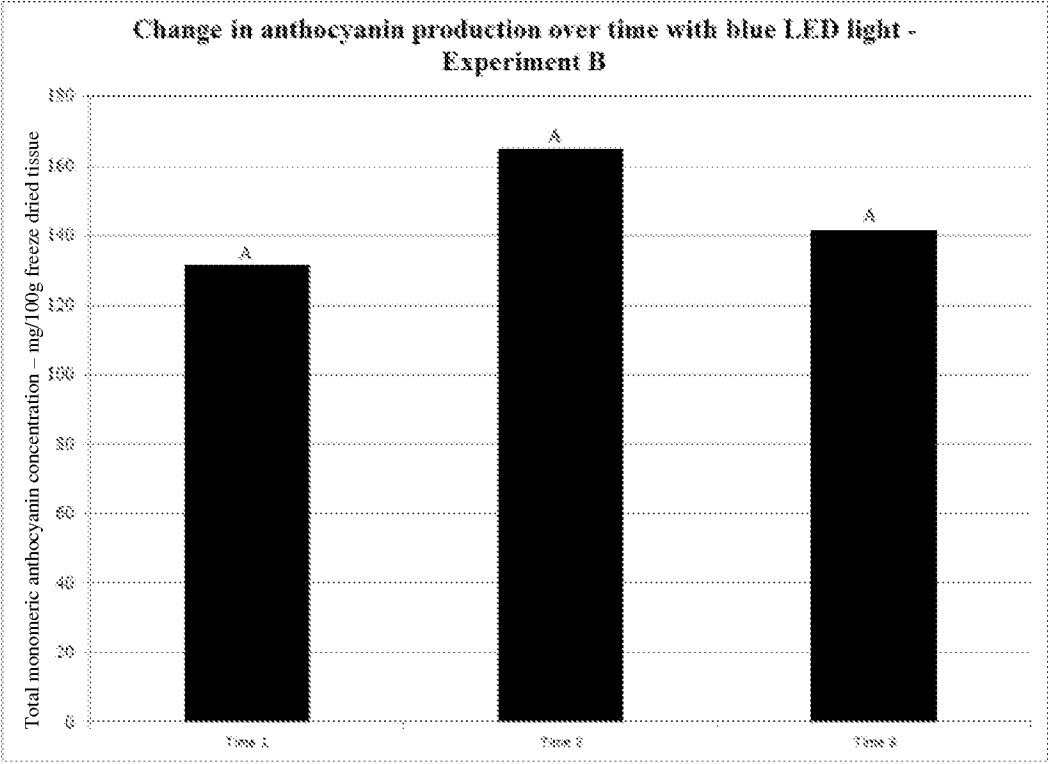


FIG. 9

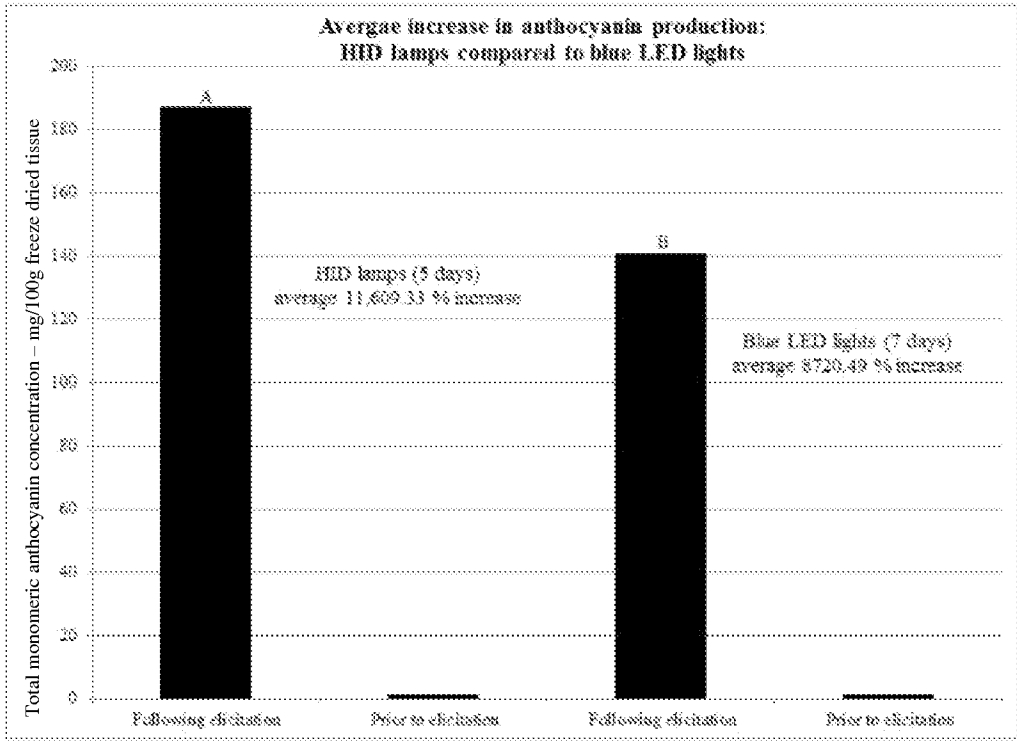


FIG. 10

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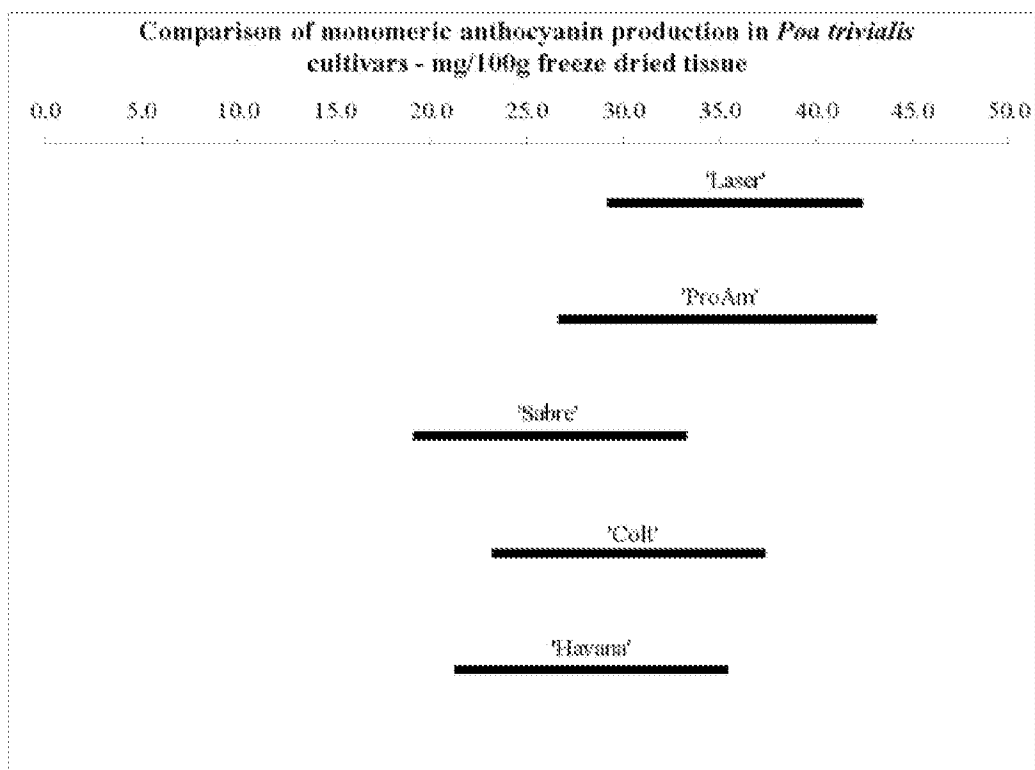


FIG. 11

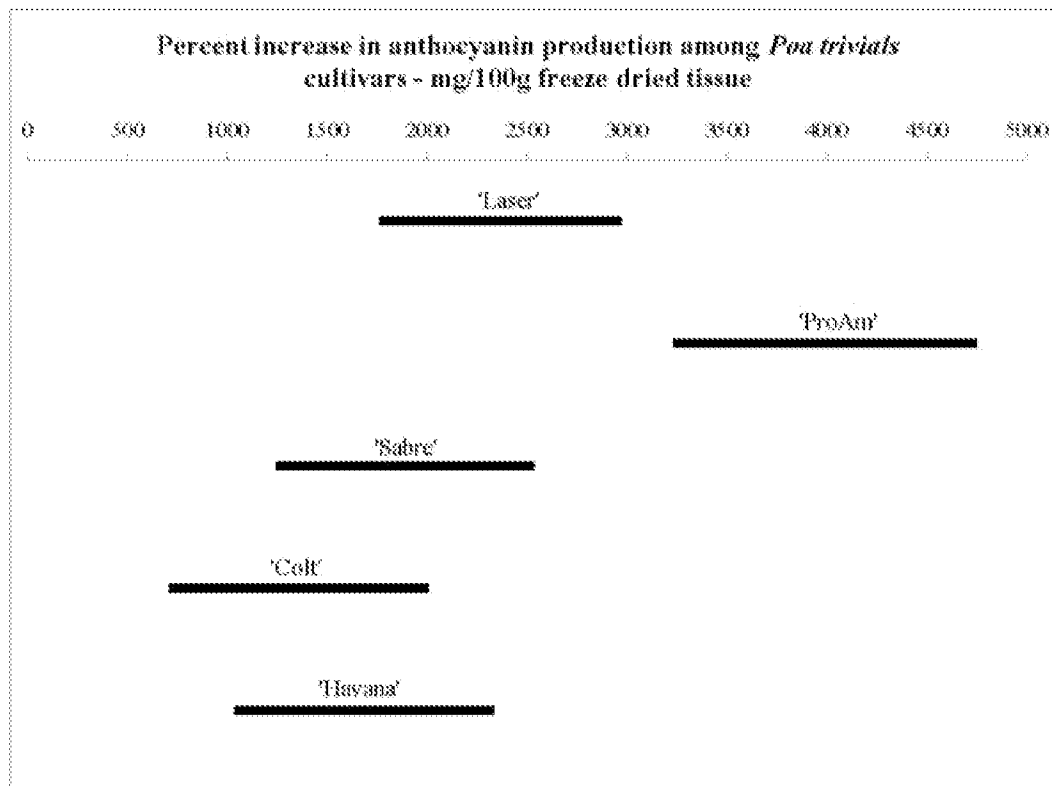


FIG. 12

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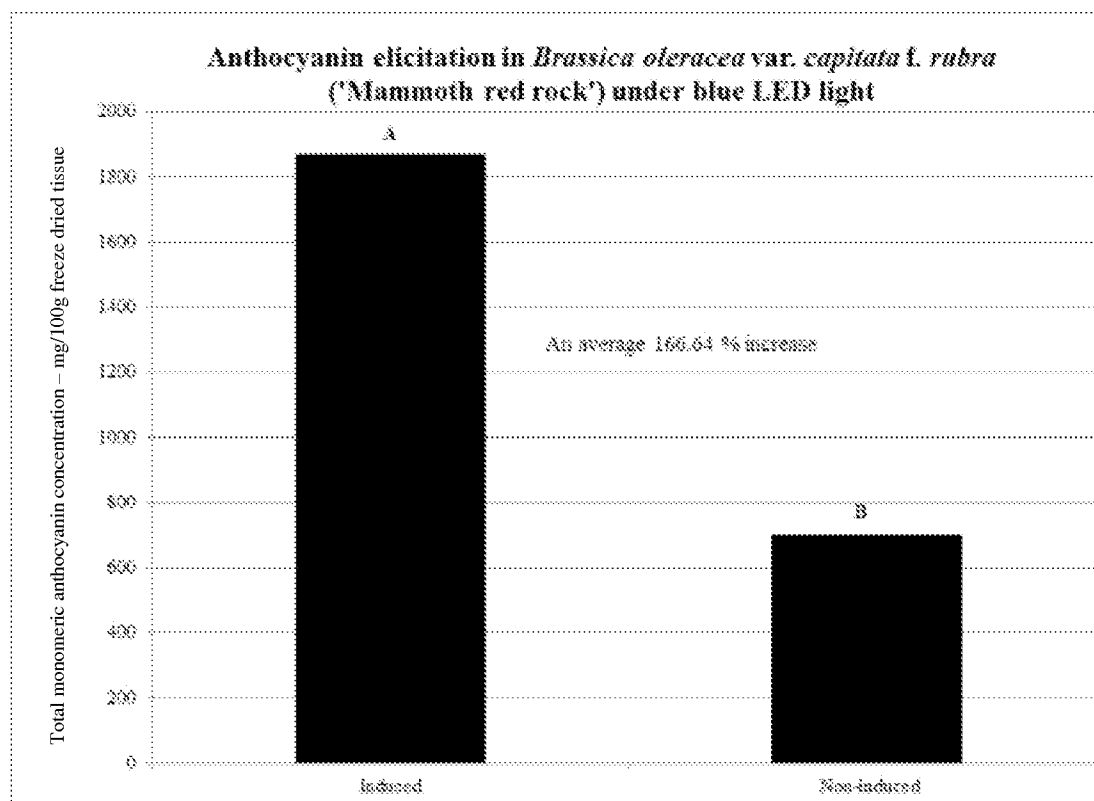


FIG. 13

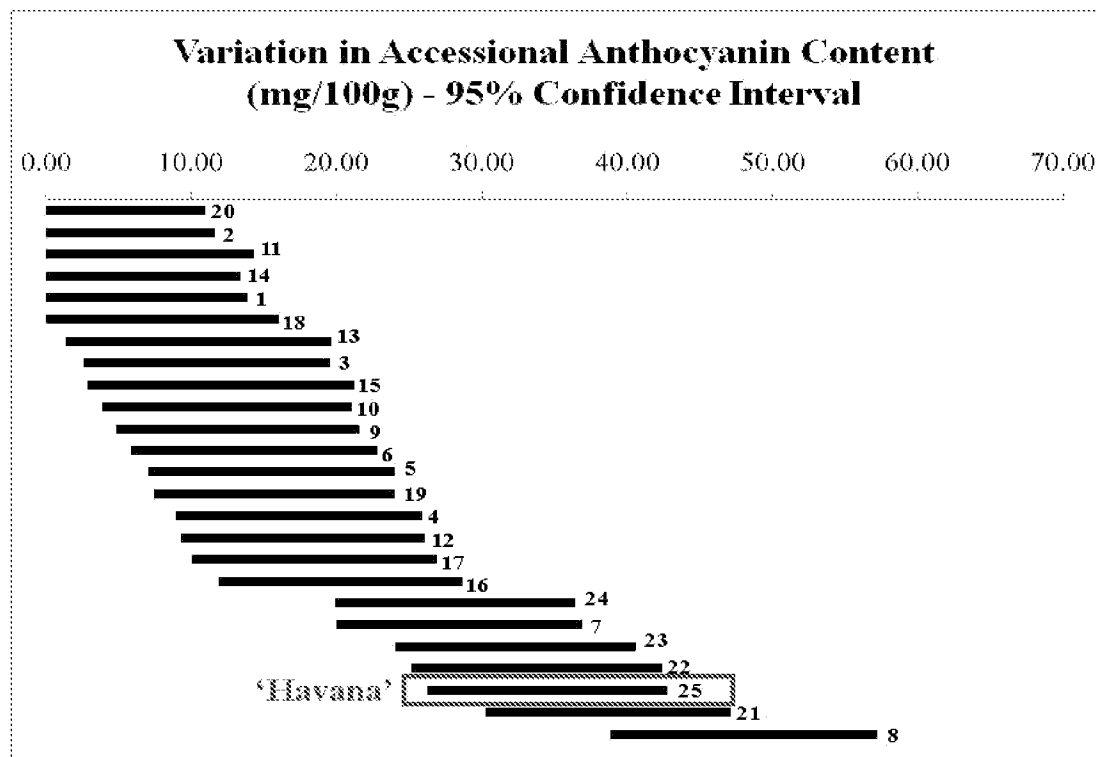


FIG. 14

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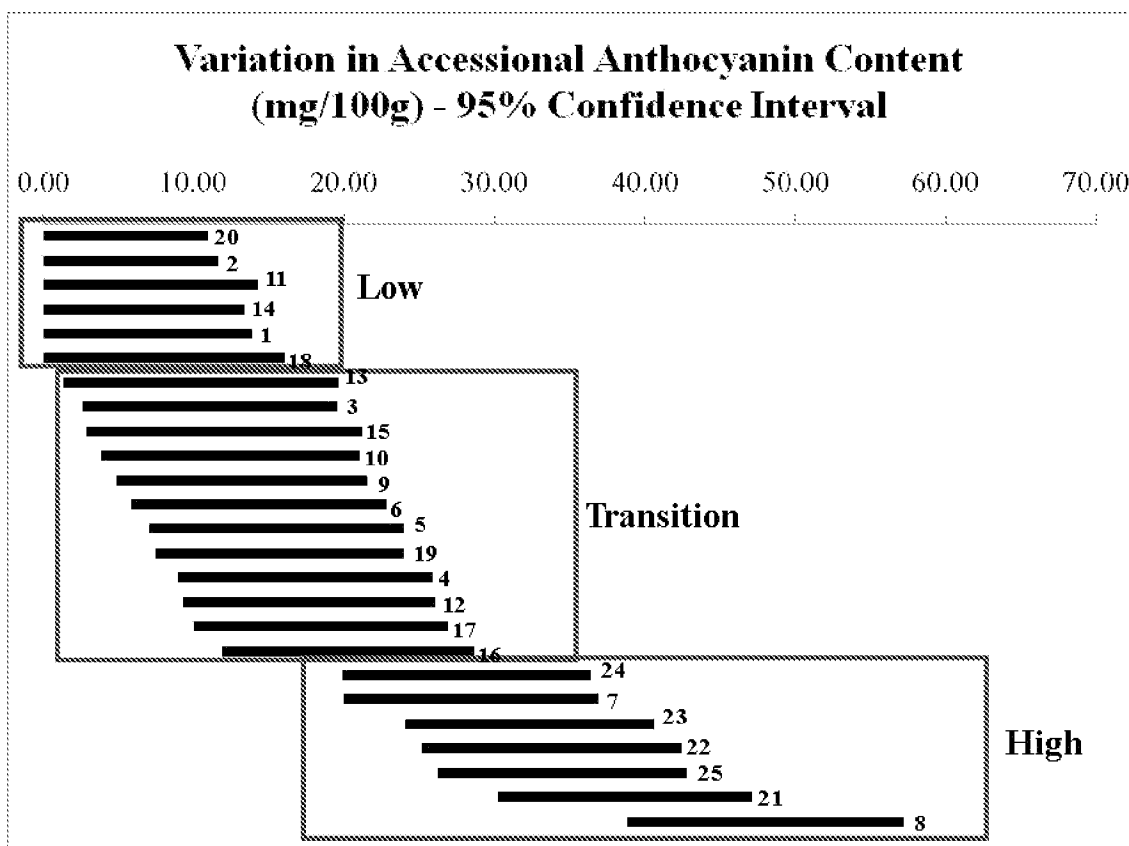


FIG. 15

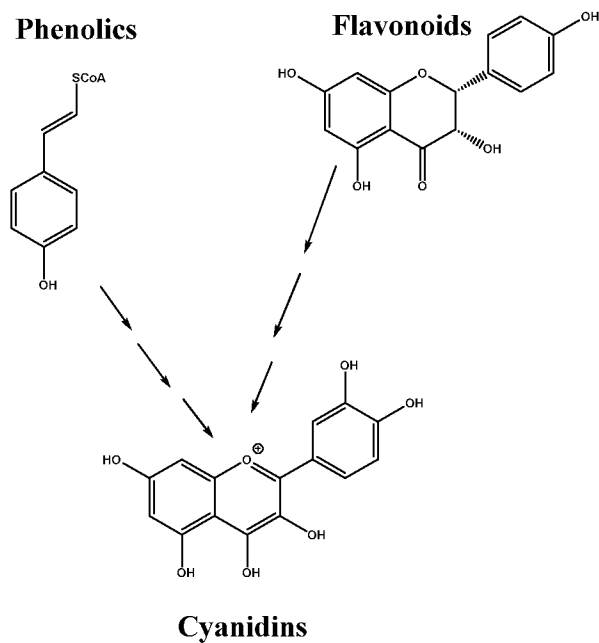


FIG. 16

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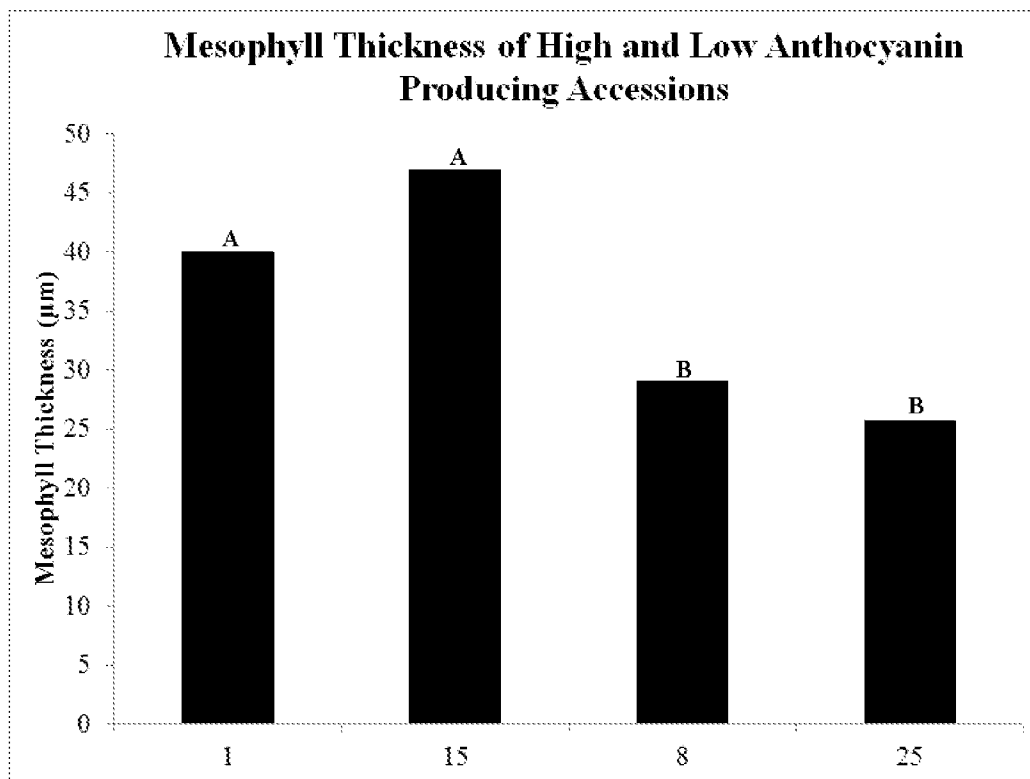


FIG. 17A

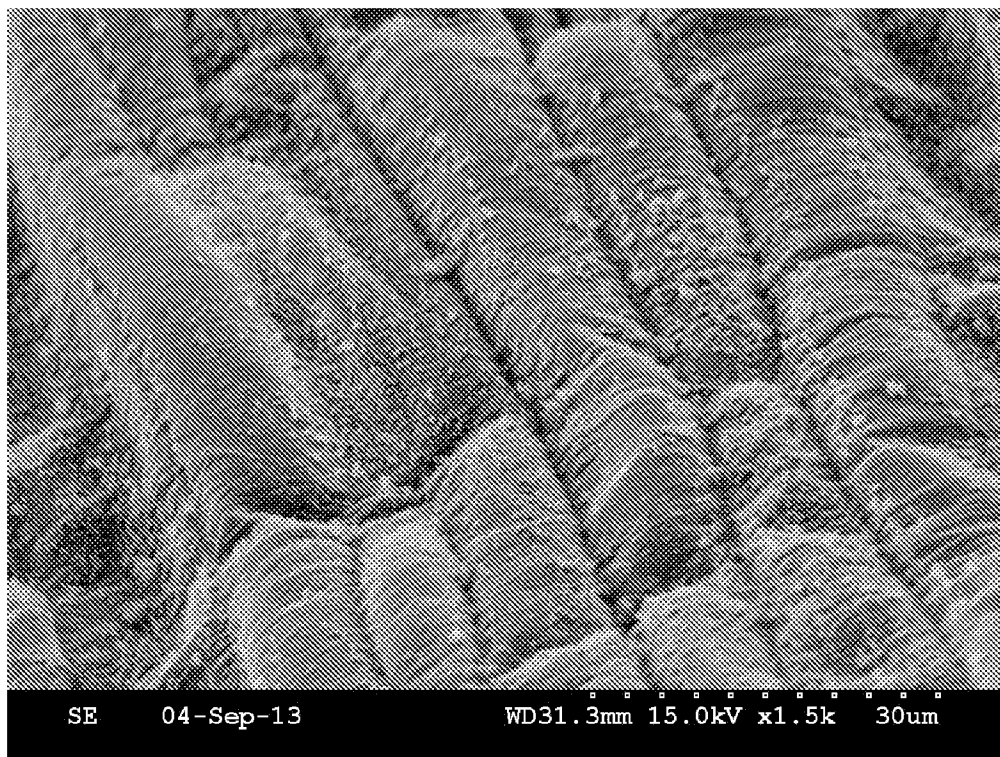


FIG. 17B

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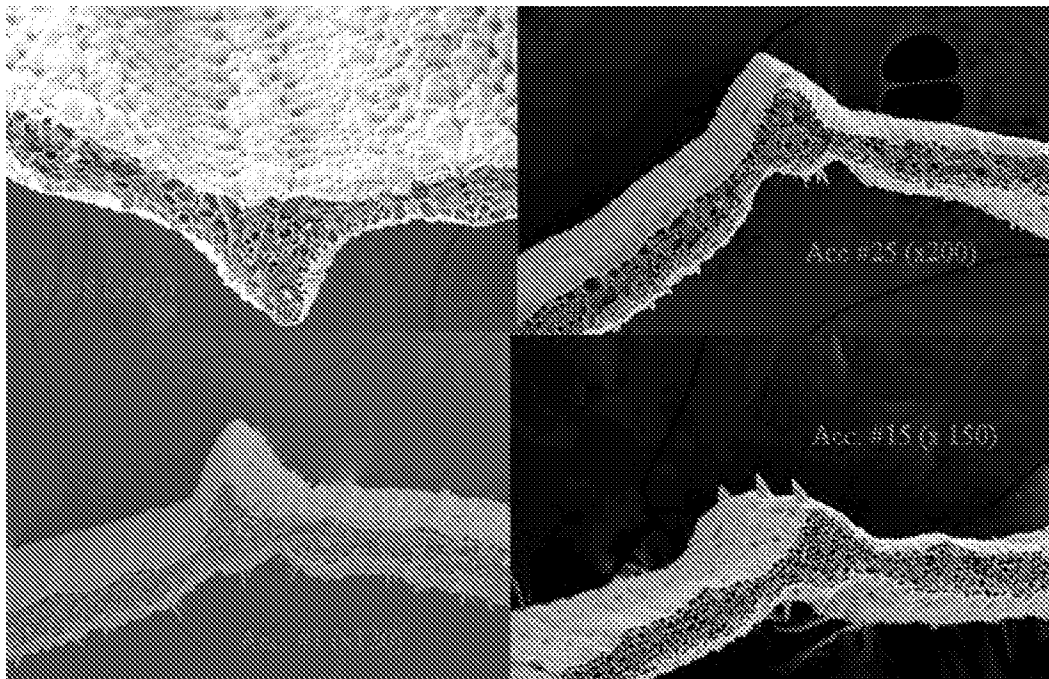


FIG. 17C

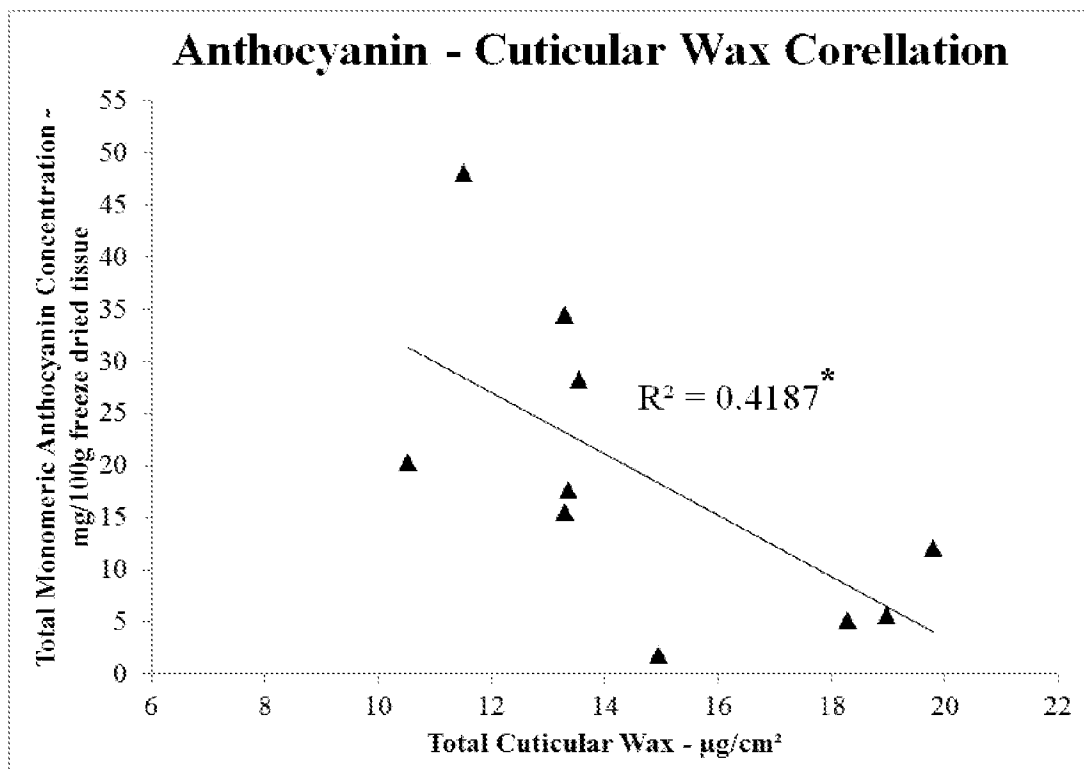


FIG. 18A

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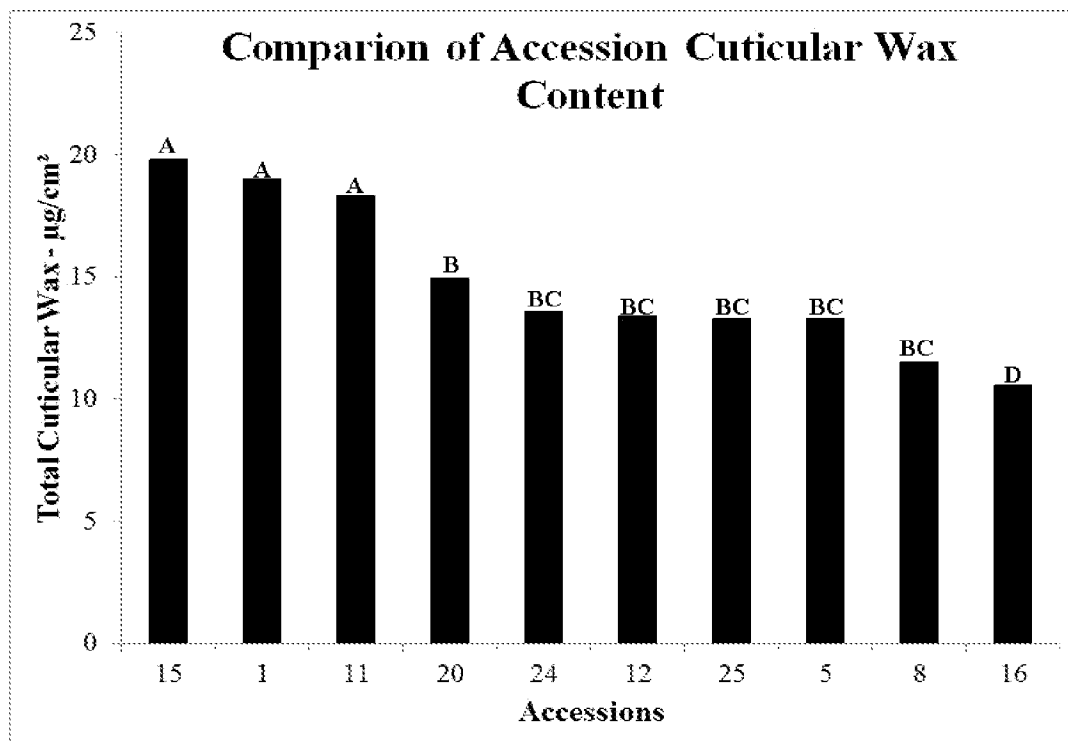


FIG. 18B

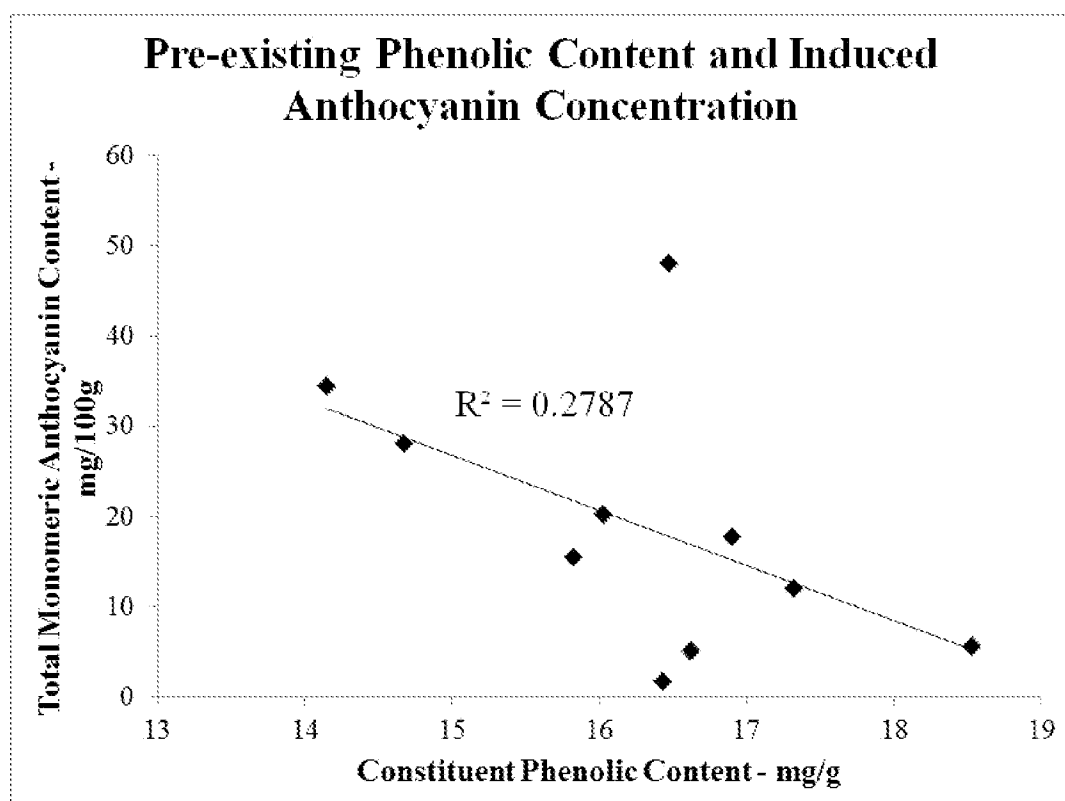


FIG. 19A

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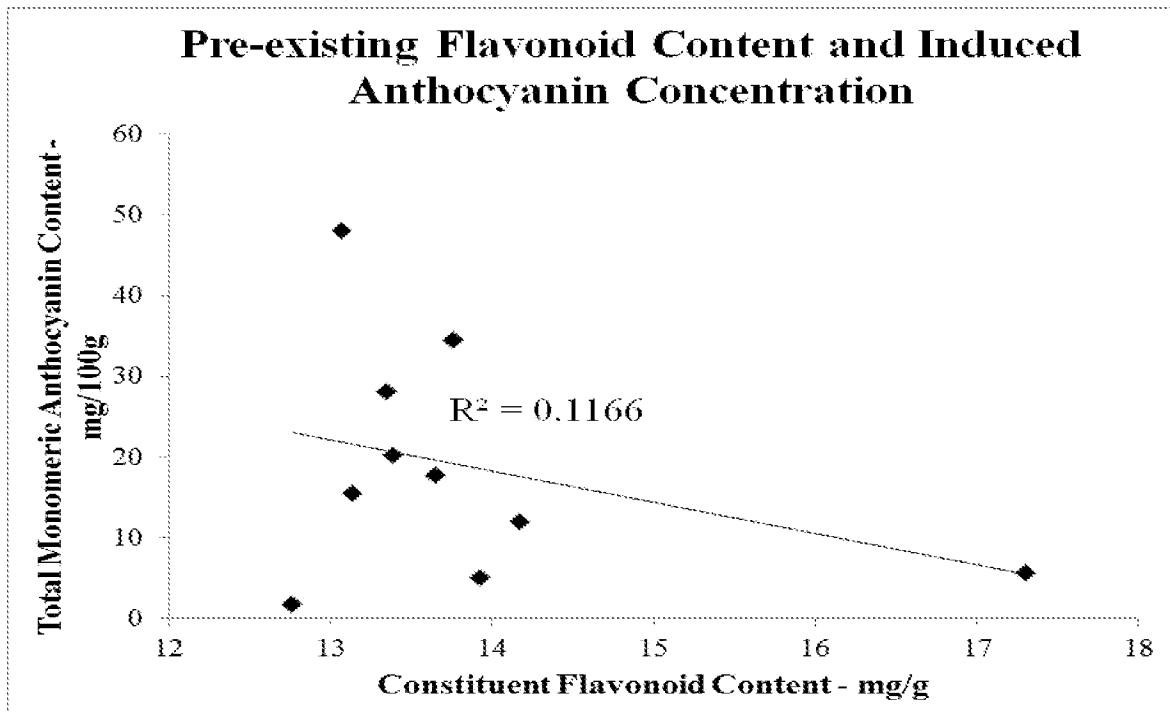


FIG. 19B

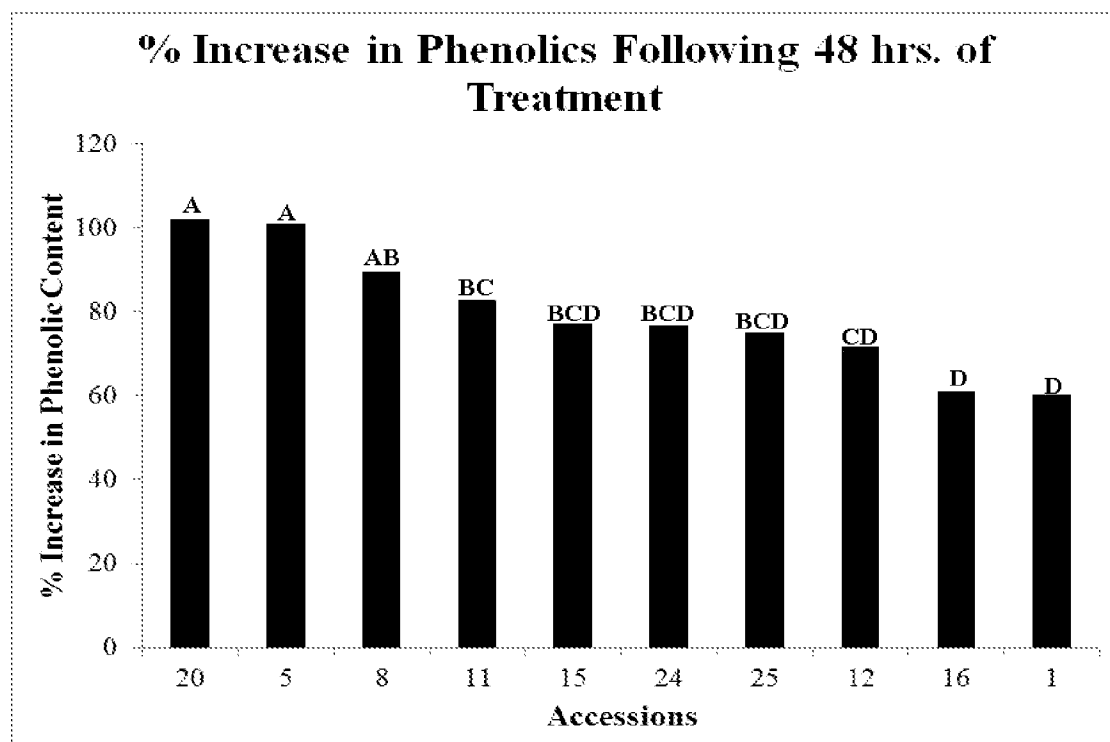


FIG. 20A

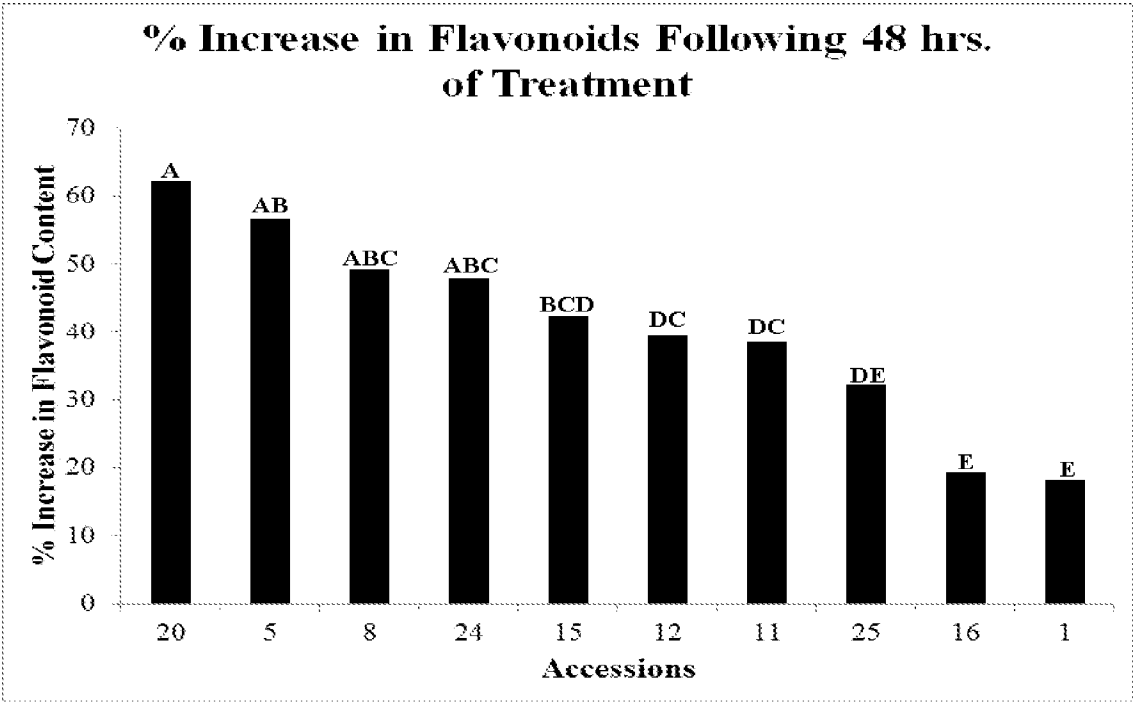


FIG. 20B

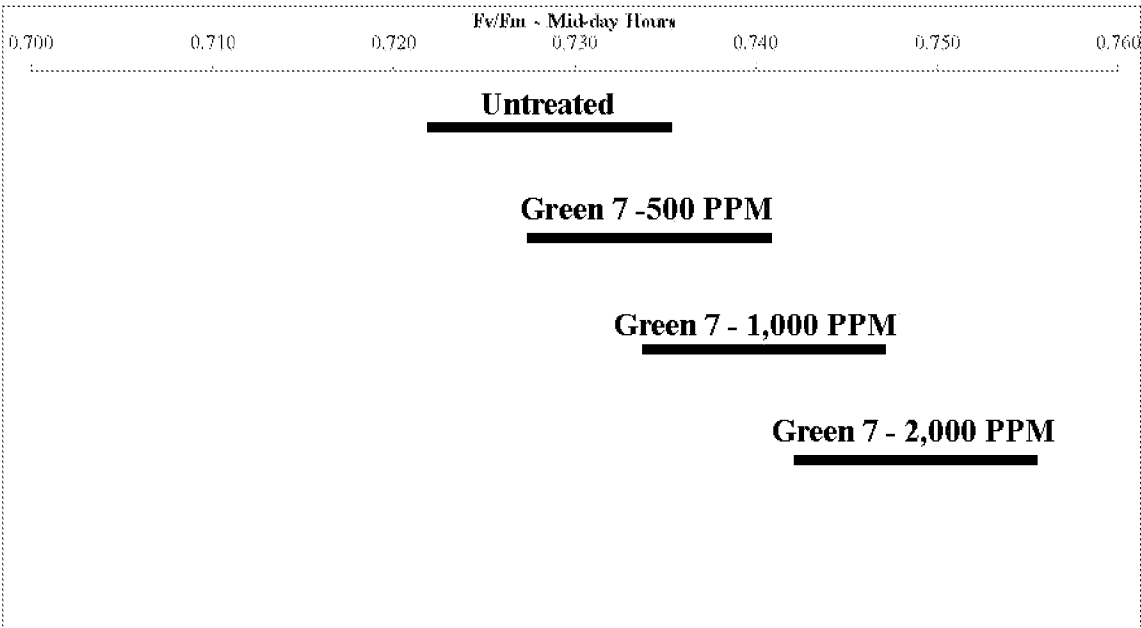


FIG. 21

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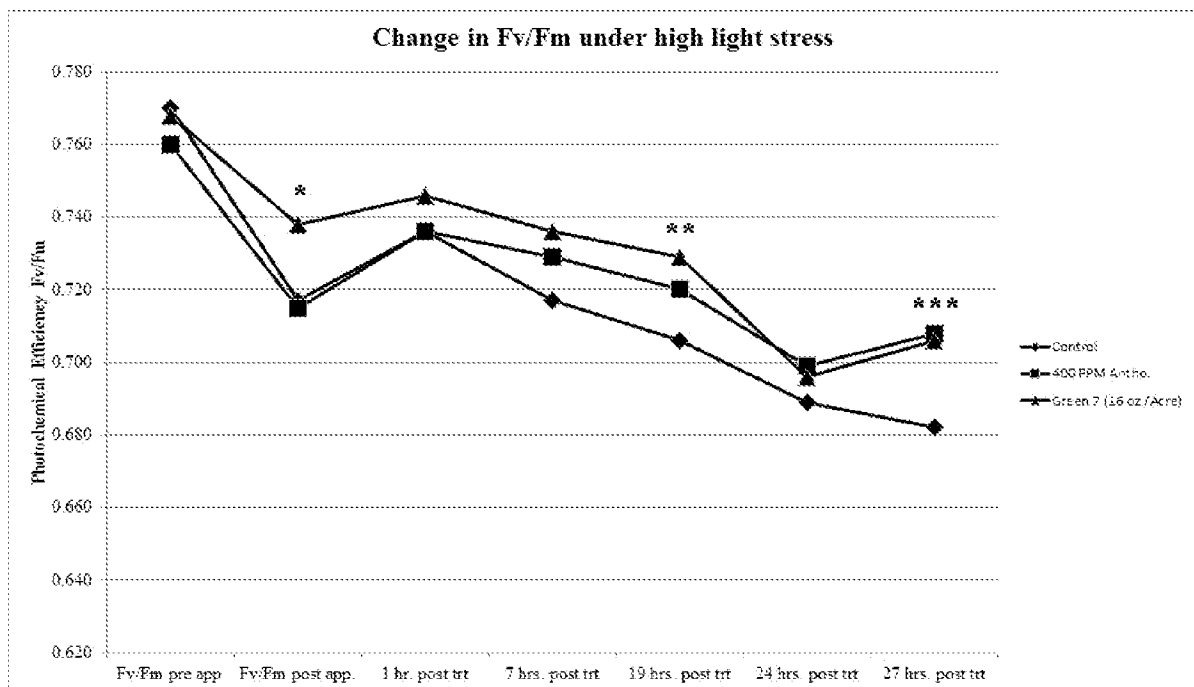


FIG. 22

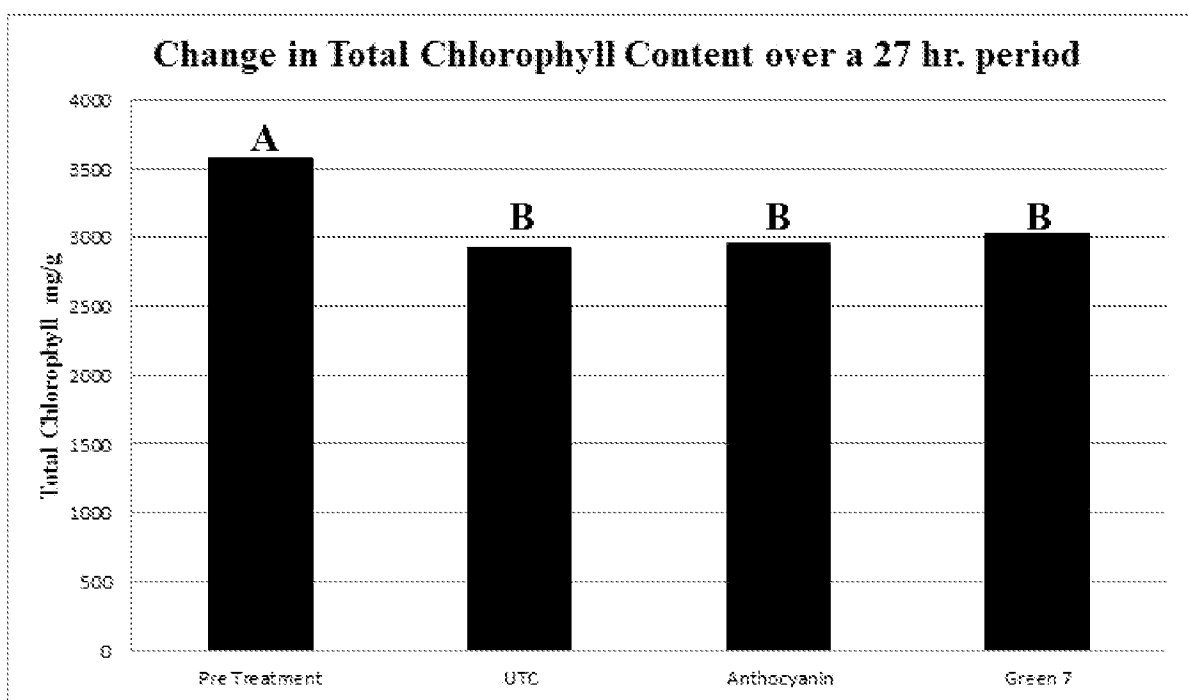


FIG. 23

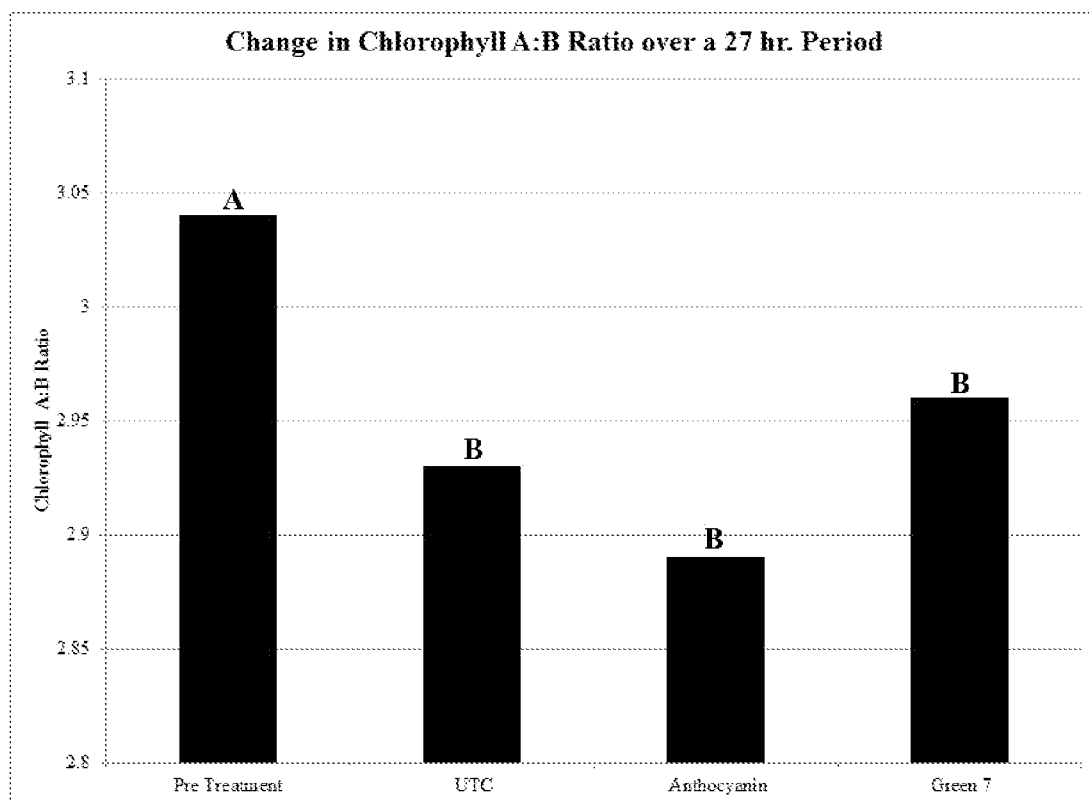


FIG. 24

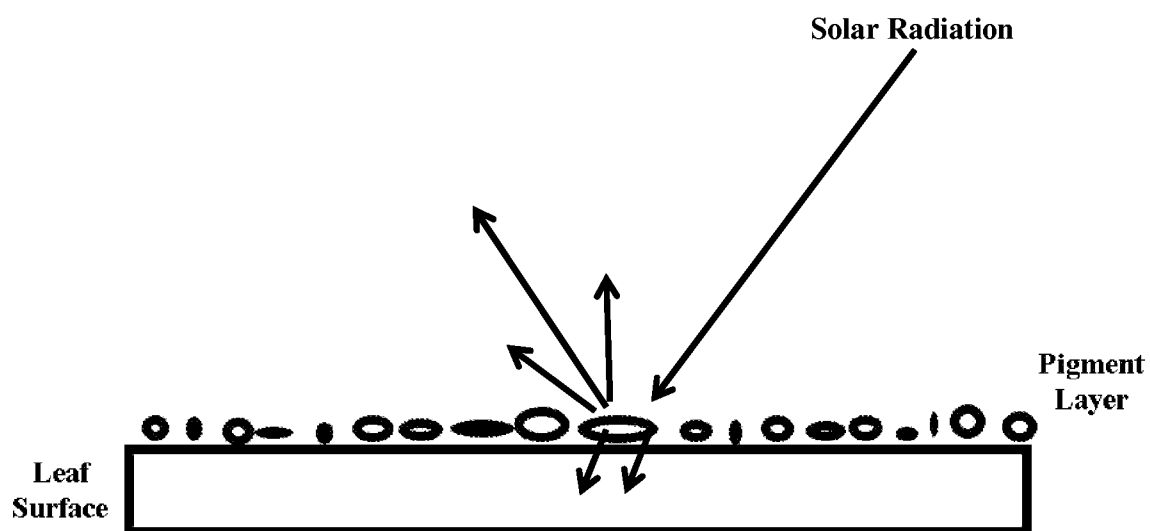


FIG. 25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US13/68262

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01H 1/02; C12N 5/04, 15/82 (2014.01)

USPC - 800/278, 298, 282

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A01H 1/02, 1/06, 5/00; C12N 5/04, 15/82, 15/29; C12Q 1/68, C07K 14/415; A01G 7/04 (2014.01)

USPC: 800/263, 278, 264, 265, 301, 279, 300, 298, 284, 282, 290, 302, 270, 281; 362/231; 435/419; 536/23.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google/Google Scholar; PubMed; ProQuest; anthocyanin, plant, 'light stress', 'osmotic stress', LED, HID, temperature, white light, blue light, flux, red cabbage, turf, turfgrass, 'light intensity'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2010/0162427 A1 (RIECHMANN, JL et al.) June 24, 2010; paragraphs [0010], [0013], [0043], [0054], [0077], [0158], [0232], [0267], [0342], [0351]-[0354], [0362], [0371], [0372], [0383], [0403], [0419], [0471], [0473], [0475], [0550], [0668]; Table 7; Claims 3, 7, 8	1, 8-10, 15-18, 27, 28, 30 ----- 6, 11-13, 29
X --- Y	KORTSTEE, AJ et al. Anthocyanin Production As A Potential Visual Selection Marker During Plant Transformation. Transgenic Research. 22 February 2011, Vol. 20, No. 6; pages 1253-1264; page 1254, column 2, paragraph 4 to page 1255, column 1, paragraph 2; page 1257, column 1, paragraph 2 to column 2, paragraph 3; page 1257, figure 3; pages 1261, column 2, paragraphs 2-6. DOI 10.1007/s11248-011-9490-1.	1, 3, 4, 7, 14, 31, 32, 34 ----- 5, 6, 19, 21-26, 33
X	WO 2002/000902 A2 (BOREVITZ, J et al.) January 3, 2002; page 3, lines 30-35; page 6, lines 1-15; page 8, lines 2-25; page 11, lines 1-5; page 11, lines 9-16; page 13, lines 18-23; Claims 29-31, 39, 46	1, 15, 17, 20
Y	PIETRINI, F et al. Anthocyanin Accumulation In The Illuminated Surface Of Maize Leaves Enhances Protection From Photo-Inhibitory Risks At Low Temperature, Without Further Limitation To Photosynthesis. Plant, Cell & Environment. 19 September 2002, Vol. 25, No. 10; pages 1251-1259; page 1252, column 1, paragraph 1; page 1253, column 1, paragraph 2 to column 2, paragraph 4. DOI: 10.1046/j.1365-3040.2002.00917.x.	5, 19, 23, 24
Y	US 7611898 B2 (WANG, Z et al.) 03 November 2009; column 1, lines 63-67; column 2, lines 1-19; column 5, lines 8-25; column 14, lines 41-45	11-13
Y	US 2008/0298052 A1 (HURST, WE et al.) December 04, 2008; paragraphs [0011], [0036]	21, 22, 25



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 March 2014 (26.03.2014)

Date of mailing of the international search report

22 APR 2014

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer:

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US13/68262

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOHKAN, M et al. Blue Light-Emitting Diode Light Irradiation Of Seedlings Improves Seedling Quality And Growth After Transplanting In Red Leaf Lettuce. HortScience. 01 December 2010, Vol. 45, No. 12; pages 1809-1814; page 1809, abstract; page 1813, Table 3.	26
Y	ENGELSJORD, ME et al. Effects Of Slow-Release Fertilizers On Growth And On Uptake And Leaching Of Nutrients In Kentucky Bluegrass Turfs Established On Sand-Based Root Zones. Canadian Journal of Plant Science. 01 July 1997, Vol. 77, No. 3; pages 433-444; page 434; column 2, paragraph 2; page 435, column 2, Table 2. DOI: 10.4141/P96-034.	29
Y	US 2008/0313822 A1 (BERTOLDO DE BARROS, RC et al.) December 25, 2008; paragraphs [0002], [0012]-[0016], [0027], [0163]-[0167]; Table 1A	33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US13/68262

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

---Please See Supplemental Page---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Groups I+: Claims 1, 3-34, Kentucky Bluegrass *Poa pratensis* turfgrass species

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

-***-Continuation of Box No. III: Observations Where Unity Of Invention Is Lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1 and 3-34 are directed toward a method for increasing anthocyanin production in a plant comprising: a) selecting a plant; b) cultivating the plant; c) applying stress to the plant, wherein the stress comprises: subjecting the plant to light stress comprising: intense synthetic light and long duration light exposure; d) harvesting plant tissue; and e) repeating steps b, c, and d.

Group II: Claims 2 and 35-38 are directed toward a method for eliciting anthocyanin production in a plant comprising: applying light to the plant, wherein the light color is substantially white or blue, wherein the light intensity is at least 400 micro-mol m⁻²s⁻¹(-01), for at least 100 continuous hours, and wherein the elicitation produces at least 50 mg of anthocyanins per 100 g foliage.

The inventions listed as Groups I+-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a method for increasing anthocyanin production in a plant comprising: a) selecting a plant; b) cultivating the plant; c) applying stress to the plant, wherein the stress comprises: subjecting the plant to light stress comprising: intense synthetic light and long duration light exposure; d) harvesting plant tissue; and e) repeating steps b, c, and d, which are not present in Group II, the special technical features of Group II including a method for eliciting anthocyanin production in a plant comprising: applying light to the plant, wherein the light color is substantially white or blue, wherein the light intensity is at least 400 micro-mol m⁻²s⁻¹(-01), for at least 100 continuous hours, and wherein the elicitation produces at least 50 mg of anthocyanins per 100 g foliage.

Groups I+ share the technical features including a method for increasing anthocyanin production in a plant comprising: a) selecting a plant; b) cultivating the plant; c) applying stress to the plant, wherein the stress comprises: subjecting the plant to light stress comprising: intense synthetic light and long duration light exposure; d) harvesting plant tissue; and e) repeating b, c, and d; and a method of plant treatment for the elicitation of anthocyanin production in a plant, wherein a majority of the above-ground plant tissue is harvested at least once in a single growing season, the treatment comprising: cultivating the plant; applying stress, wherein the stress comprises: subjecting the plant to light stress comprising: intense light and long duration light exposure; harvesting the majority of the above-ground plant tissue; and extracting anthocyanins from the tissue. Groups I+-II share the technical features including a method for eliciting anthocyanin production in a plant comprising: applying light to the plant.

However, these shared technical features are previously disclosed by the publication entitled 'Ultraviolet Light And Its Effect On Germination, Growth, Physiology And Responses Of Cool Season Grasses' by Nangle (hereinafter 'Nangle'). Nangle discloses a method for increasing anthocyanin production in a plant (abstract; (iii)) comprising: 1) selecting a plant (abstract; (iii)); b) cultivating the plant (Conclusion; pages 129-130); c) applying stress to the plant (abstract; page 38), wherein the stress comprises: subjecting the plant to light stress (page 44, first paragraph) comprising: intense synthetic light (page 79, figure 3.1) and long duration light exposure (page 95, first paragraph); d) harvesting plant tissue (page 151, second paragraph); and e) repeating b, c, and d (pages 46, 48; four replications of each cultivar were used in two repeated trials); and a method of plant treatment for the elicitation of anthocyanin production in a plant (abstract; (iii)), the treatment comprising: cultivating the plant (Conclusion; pages 129-130); applying stress (abstract; page 28), wherein the stress comprises: subjecting the plant to light stress (page 44, first paragraph) comprising: intense light (page 79, figure 3.1) and long duration light exposure (page 95, first paragraph); harvesting the above-ground plant tissue (page 151, second paragraph); wherein a majority of the above-ground plant tissue is harvested at least once in a single growing season (page 44, second paragraph to page 46, third paragraph; two separate turfgrass trials; six turfgrass varieties; six cultivars of colonial bentgrass were evaluated); and extracting anthocyanins from the tissue (page 17, second paragraph); and a method for eliciting anthocyanin production in a plant (abstract, (iii)) comprising: applying light to the plant (page 44, first paragraph); and harvesting the majority of the above-ground plant tissue (page 44, second paragraph to page 46, third paragraph, two separate turfgrass trials; six turfgrass varieties; six cultivars of colonial bentgrass were evaluated).

Since none of the special technical features of the Groups I+-II invention is found in more than one of the inventions, and since all of the shared technical features are disclosed by the Nangle reference, unity of invention is lacking.