JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Antioxidant Activity of Selected Stilbenoids and Their Bioproduction in Hairy Root Cultures of Muscadine Grape (*Vitis rotundifolia* Michx.)

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Supporting Information

ABSTRACT: Stilbenoids are polyphenolic phytoalexins with health-related properties in humans. Muscadine grape (*Vitis rotundifolia*) hairy root cultures were established via *Agrobacterium rhizogenes*-mediated transformation, and the effects of growth regulators (3-indolebutyric acid and 6-benzylaminopurine) and methyl jasmonate (MeJA) on stilbenoid production were studied. Twenty-one-day-old hairy root cultures were treated with 100 μM MeJA for 24 h, and then the stilbenoids were extracted from the medium and tissue with ethyl acetate and analyzed by HPLC. Resveratrol, piceid, and ϵ-viniferin were observed preferentially in tissue, whereas piceatannol was observed only in medium. Growth regulators did not affect the yield of stilbenoids, whereas higher levels were found upon treatment with MeJA. Stilbenoids identified in the hairy root cultures were analyzed for their radical scavenging capacity showing piceatannol and ϵ-viniferin as the strongest antioxidants. Muscadine grape hairy root cultures were demonstrated to be amenable systems to study stilbenoid biosynthesis and a sustainable source of these bioactive compounds.

KEYWORDS: *resveratrol, piceatannol, piceid, ϵ-viniferin, stilbenoid, hairy roots, muscadine grape, Vitis rotundifolia, elicitation, antioxidant*

INTRODUCTION

Muscadine grape (*Vitis rotundifolia* Michx.), a species from the Vitaceae family, is native to the southeastern region of North America, where it is widely distributed. The term muscadine derives from the muscat grape, which is a European grapevine cultivar with a characteristic berry scent similar to that of the wild grapes that early European settlers found in North America. This species is also an important commercial crop used in fresh consumption, food additives, dietary supplements, and the wine industry. Unlike other grape species, the inherent higher resistance of muscadine grape to pathogens enables a sustainable production in the region.[1−3] This species currently comprises over 50 cultivars such as Fry, Noble, Carlos, Triumph, and Bountiful, among many others.[4] It has been reported that muscadine grape skins and seeds contain significant concentrations of stilbenoids[5] and possess one of the highest antioxidant levels among fruits.[6] Accordingly, a growing number of studies have attributed beneficial health properties to the stilbenoid content of muscadine grape fruits.[6−8]

Resveratrol (Figure 1) is a polyphenolic compound that belongs to the stilbenoid group. Two isomers, *cis* and *trans*, are known. The *trans* form can undergo isomerization to the *cis* form by light exposure.[9] Resveratrol isoforms can also be bound to glucose, forming piceid,[10] or oligomerized to form viniferins by peroxidation.[11] Piceatannol, a hydroxylated analogue of resveratrol, has been also found in red wine (for a review on stilbenoids, see Shen et al.[12]).

Stilbenoids have been found in several taxonomically unrelated plant species[13] and are believed to function as phytoalexins.[14,15] In grapevine, stilbenoids are produced as a response to infection or injury.[16] Furthermore, these compounds possess antioxidant properties that may provide the species supporting this metabolic pathway protection against oxidative stress and other environmental challenges such as exposure to UV radiation and ozone.[17,18]

The research on resveratrol and its related compounds has grown exponentially since 1997 when it was shown to have cancer chemopreventive activity against three major stages of carcinogenesis: inhibiting cellular events associated with tumor initiation, promotion, and progression.[19] Several studies have been done since then on the antioxidant, anticancer, antibacterial, and anti-inflammatory properties of resveratrol and its analogues in vitro and in vivo (for a review on resveratrol and its biological targets and functional activity see Pervaiz and Holme[20]). From the 33 plant families that are known to include stilbenoid-producing species, the genus *Vitis* has been the most studied because high levels of these polyphenols can be found in its fruits and derived products such as red wine.[12,21] For instance, the presence of resveratrol in wine has been used to explain the so-called "French paradox", in which the moderate consumption of wine in the Mediterranean diet is presumed to reduce the risk of cardiovascular diseases.[22]

Over the past years, hairy root cultures produced via *Agrobacterium rhizogenes*-mediated transformation have emerged as an ideal biological system to study secondary

Special Issue: Human Health and Transgenic Crops

Received: February 20, 2013
Revised: May 8, 2013
Accepted: May 13, 2013
Published: May 13, 2013

dx.doi.org/10.1021/jf400760k | J. Agric. Food Chem. 2013, 61, 11744−11758

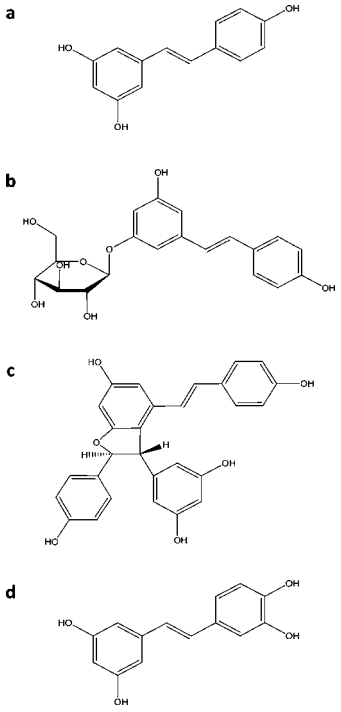

Figure 1. Chemical structures of stilbenoids produced in hairy root cultures of muscadine grape: (a) resveratrol; (b) piceid; (c) *ε*-viniferin; (d) piceatannol. All compounds are shown in their *trans* isomers.

metabolism.[23,24] Furthermore, hairy roots show higher genetic and biochemical stability when compared to cell cultures, and their ability to synthesize their own hormones makes possible their culture in media without growth regulators, the presence of which is not desirable in end products.[25] Hairy roots, as other plant tissues, respond to biotic and abiotic stressors by up-regulating distinct specialized metabolic pathways. This approach has been used to obtain high yields of several medicinal compounds.[26]

Hairy roots carry in their genome the *rol* genes as a result of the transfer of the plasmidic T_L-DNA from *A. rhizogenes*. The *rolA*, *rolB*, and *rolC* genes have been previously related to plant growth. Moreover, in recent studies it has been proposed that they are involved in the induction of the synthesis of secondary metabolites in transformed cells. However, the regulatory mechanisms are still unclear (for reviews on the functions and applications of the *rol* genes in plant secondary metabolism see Bulgakov[27] and Bulgakov et al.[28]). A study on the *rolB* gene-mediated induction of resveratrol in calli cultures of *Vitis amurensis* reported accumulation of 2 orders of magnitude higher than in cell suspension cultures.[29] More recently, using cell cultures of *V. amurensis*, these findings were correlated with an expression enhancement by *rolB* of phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*), gene families involved in the biosynthesis of resveratrol.[30] The effect of the *rolC* gene on the induction of other types of compounds such as the ginsenosides in transgenic hairy roots of *Panax ginseng* showed a 3-fold increase in their biosynthesis compared to the control.[31] Similarly, Dubrovina et al.,[32] working on callus cultures transformed with the *rolC* gene, reported an accumulation of resveratrol production of 1 order of magnitude higher than in cell suspension cultures that was attributed to the expression of *PAL* and *STS* genes.

Jasmonic acid (JA), a stress-related growth regulator, and its esters, particularly methyl jasmonate (MeJA), have been widely used in promoting the biosynthesis of inducible secondary metabolites.[26] For instance, resveratrol production can be stimulated in grapevine (*Vitis vinifera*) cell cultures treated with MeJA.[33,34] Laminarin[35] and cyclodextrins[36] among other elicitors have been also used to induce the stilbenoid response in *V. vinifera* cell suspension cultures.

Research on *V. rotundifolia* cell suspension cultures has been performed to study the regeneration of plants[37] and the expression of the chalcone synthase gene family involved in flavonoid biosynthesis.[38] However, no detailed studies have been conducted on stilbenoid biosynthesis with cell suspension cultures of this species. Furthermore, the use of these systems has as a major drawback the continued use of growth regulators, which tends to induce somaclonal variation and affect the biosynthetic pathways of the compounds of interest (for a review on somaclonal variation, see Larkin and Scowcroft[39]). This led us to believe that the transformation of muscadine grape by *A. rhizogenes* could result in hairy root lines with the potential to synthesize stilbenoids for research and pharmaceutical applications.

As far as we know, this is the first study describing the establishment of hairy root cultures of muscadine grape and their stilbenoid profile. This system provides a controlled and sustainable tissue culture for characterizing the mechanisms that affect the biosynthesis, accumulation, and metabolism of stilbenoids in muscadine grape and enables the possibility of identifying additional bioactive compounds. The culture parameters that ensure reproducibility of this model system for studying these phenolic compounds are discussed. Furthermore, we describe the antioxidant capacities of purified stilbenoids that can be found in the medium and tissue of these hairy root cultures.

MATERIALS AND METHODS

Establishment of Hairy Root Cultures. Fresh berries of the muscadine grape cvs. Fry and Noble (provided by Dr. Stephen Stringer, USDA ARS, Poplarville, MS, USA) were cut in half and the seeds removed. Seeds were rinsed in 50% Clorox solution for 3 min, rinsed in running tap water, and quickly air-dried. Then the seeds were surface disinfested in 50% bleach + 0.001% Ivory for 15 min, followed by rinses for 2 min (once) and for 7.5 min (twice) in successive changes of sterile water. The seeds were immediately plated on germination media ($^1/_{10}$ strength Gamborg's B5 medium[40] with 3% sucrose, pH 5.7) in 100 × 25 mm sterile Petri dishes or on sterile peat pellets placed in sterile Magenta boxes. All seeds were then stored for a minimum of 8 weeks at 4−6 °C in the dark to satisfy stratification requirements. At the end of this period the Petri dishes and Magenta boxes with peat pellets were moved into incubators at 28 °C with constant light at 130 μmol^{-2} s^{-1} light intensity.

Germination occurred approximately 14 days after the seeds had been placed in the warm lighted incubator. After emergence of the radicle, seedlings were held until the first true leaves began expanding. Explants were harvested from 8−10 seedlings with expanding first leaves. The explants that were tested included leaf blade, hypocotyl, cotyledon, and leaf with petiole. These were placed on semisolid Dunstan and Short (BDS) medium[41] containing 4 g/L Phytagel (Sigma). Explants were inoculated with *A. rhizogenes* ATCC 15834 (Figure 2a) as previously described by Medina-Bolivar and Cramer[42] via epidermal incisions and incubated in the dark at 28 °C for 21−28 days. Putative hairy roots that emerged from the inoculated sites were isolated and transferred to semisolid BDS medium containing 600 mg/L cefotaxime. Root initials were subcultured on this medium twice at 2 week intervals and then transferred to the same media without antibiotics.

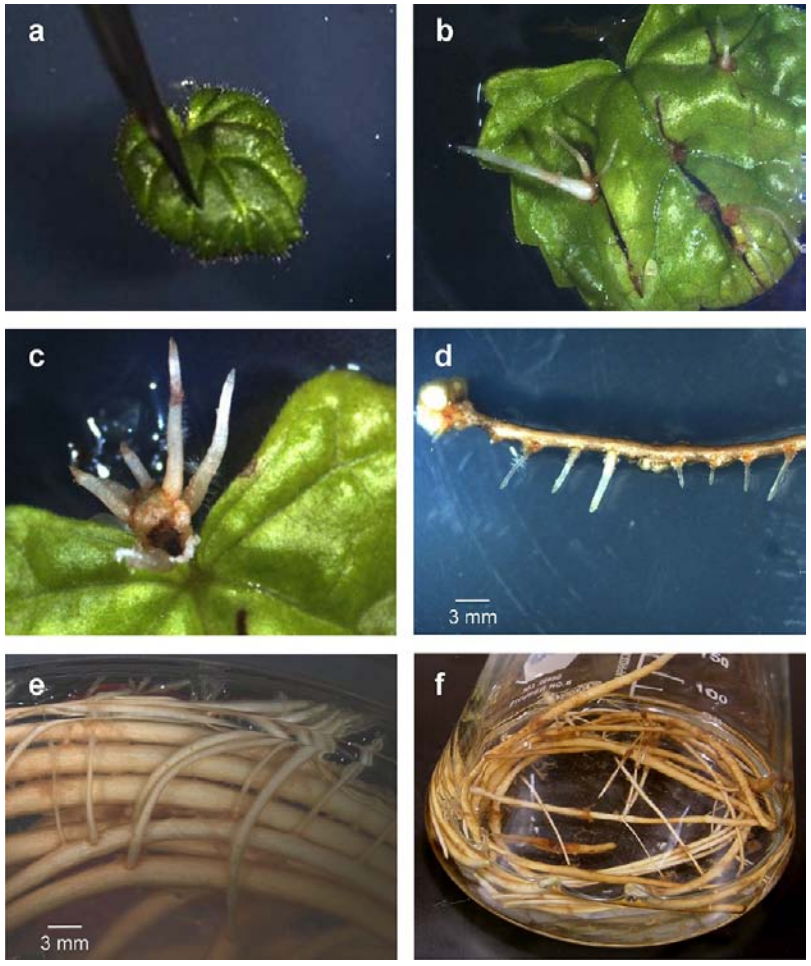

Figure 2. Establishment of hairy root cultures of muscadine grape: (a) infection of a leaf explant of cv. Fry with *Agrobacterium rhizogenes*; (b) early response from the infection visualized as a small callus from which hairy roots are formed later; (c) leaf showing several putative hairy roots from the inoculation site; (d) isolated mature hairy root on BDS+GR medium with cefotaxime; (e) hairy roots grown on Petri dishes; (f) established hairy root cultures in liquid BDS medium.

Hairy root cultures were allowed to grow on semisolid BDS medium until sufficient biomass accumulated before transfer into liquid medium. Hairy root liquid cultures were first initiated in 250 mL Erlenmeyer flasks with 50 mL of BDS medium. After the roots had adapted to liquid culture, they were transferred to BDS medium with growth regulators, 0.05 mg/L 3-indolebutyric acid (IBA) and 0.05 mg/L 6-benzylaminopurine (BAP), hereafter referred to as BDS+GR. These growth regulators were added to the medium prior to autoclaving. Cultures were subcultured by inoculating 5−10 pieces of root tips of 2−3 cm in length preferably showing at least one secondary root when possible.

Molecular Characterization of Hairy Roots. DNA was extracted from 200 mg of lyophilized root tissue from lines Fry-3A and Nob-2Cot using a CTAB method[43] with modifications. Tissue was placed in a 50 mL capacity tube with CTAB extraction buffer (20 mL) and mercaptoethanol (200 μL) (Sigma) followed by incubation at 65 °C for 20 min and cooled at room temperature for 10 min. Twenty milliliters of chloroform/isoamyl alcohol (24:1) (Fluka) solution was added and then mixed by gentle inversion for 5 min. Samples were centrifuged at 4000 rpm at for 15 min in a centrifuge 5810R (Eppendorf) at 4 °C. The supernatant was transferred to a clean 50 mL tube, and the previous two steps were repeated. Cold isopropanol (2/3 volumes; 15 mL) was added, and then the mixture was incubated at −20 °C for 1 h followed by centrifugation at 4000 rpm at 4 °C for 20 min to eliminate the supernatant. The pellet containing the DNA was transferred to a clean 2 mL microtube and washed with 500 μL of washing solution I (sodium acetate, ethanol 100%, and H_2O in final concentrations of 0.2 M, 75%, and 25%, respectively) followed by incubation at room temperature for 10 min and then centrifugation at 4000 rpm for 5 min at 4 °C. Precipitated DNA was transferred to a clean 2 mL microtube and washed with 300 μL of washing solution II (ammonium acetate, ethanol 100%, and H_2O in final concentrations of 10 mM, 75%, and 25%, respectively) with incubation for 1 min at room temperature. The next step included centrifugation at 4 °C for 5 min and elimination of supernatant. DNA was dissolved in 500 μL of nuclease-free water (Sigma). Incubation with 5 μL of 10 μg/mL RNase (Qiagen) at 37 °C for 30 min was performed to eliminate contaminant RNA.

PCR analyses for *rolC*, *aux1*, and *virD2* were performed according to the method of Medina-Bolivar et al.[24] using a Stratagene RoboCycler Gradient 96. PCR products were run in 1% agarose gel, and the amplicon size was verified by comparison to the TrackIt 1 kb Plus DNA Ladder (Invitrogen).

Growth Kinetics. To establish the growth kinetics of hairy roots lines Fry-3A and Nob-2Cot in BDS medium, 10 root tips (4−5 cm long with two root primordia) with an average of 0.087 g dry weight (DW) (1.14 g fresh weight (FW)) were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of BDS and BDS+GR media. The pH was adjusted to 5.8 for both types of media prior to autoclaving; however, the pH postautoclaving was recorded as 5.58 and 5.59 for BDS and BDS+GR, respectively. The cultures were grown on gyratory shakers (Innova 44R, New Brunswick Scientific) at 90 rpm and 28 °C and maintained under darkness. Three culture flasks were harvested every 3 days from day 0 to day 30. Harvested roots were rinsed three

times with tap water, and excessive water was slightly removed with paper towels. Fresh weights were determined, and the roots were placed in an oven at 60 °C for 3 days to obtain the DW. The specific growth rate (μ) was calculated as

$$\mu = \frac{\ln \frac{DW_i}{DW_0}}{\Delta t}$$

where DW_i is the average dry weight of the roots in grams at the end of the exponential growth (24 days for both BDS and 27 days for BDS +GR medium), DW_0 is the average dry weight of inoculum in grams at the start of the exponential growth (day 0), and t is the interval of time (in days) between 0 and i (24 days for BDS and 27 days for BDS+GR medium). Doubling time (T_d) was calculated as

$$T_d = \frac{\ln(2)}{\mu}$$

where μ is the calculated specific growth rate of the hairy roots.

Conductivity and pH of spent and fresh media were measured using a SevenEasy conductivity meter (Mettler Toledo) and an Accumet Basic AB15 pH meter (Fisher Scientific), respectively.

Elicitation Experiments. Hairy roots from lines Fry-3-A and Nob-2Cot were grown in 250 mL flasks with 50 mL of BDS and BDS+GR media as described above. The elicitor MeJA was dissolved in absolute ethanol (EtOH) (Sigma) and used at a final concentration of 100 μM. Elicitations were performed at day 21 of culture. Prior to elicitation, the spent medium was collected from each culture, and pH and conductivity values were recorded as described above. Fresh medium containing MeJA was added to each treatment culture, and 50 μL of absolute EtOH was added to the control cultures. All elicitations were carried out for 24 h at 28 °C under continuous darkness. Following elicitation, roots and media were collected. FW was measured, and the roots were immediately frozen with liquid nitrogen. The processed tissue was packed in previously weighed aluminum foil and lyophilized in a FreeZone 4.5 freeze-drying apparatus (Labconco). The weight of the aluminum foil and the dried tissue was recorded, and the difference from the previously recorded value for the aluminum foil was considered as the DW mass. Lyophilized tissue was kept at −80 °C for further analyses.

The pH and the conductivities of the media of the MeJA-treated and nontreated cultures were measured and recorded as previously described. All experiments were performed using three biological replicates per treatment.

Stilbenoid Extraction. Lyophilized root tissue samples were ground using a mortar and pestle until a very fine powder was achieved. The ground tissue was placed in 1 mL microcentrifuge tubes and vortexed for 1 min in ethyl acetate (EtOAC) in a ratio of 0.05 g DW tissue to 1 mL solvent. Sonication was performed afterward for 30 s followed by a centrifugation for 8 min at 16100*g* to remove the nondissolved material. Supernatant fractions were carefully pipetted and transferred to amber glass tubes (to avoid degradation by light) and evaporated to dryness under nitrogen stream using a Reacti-Vap III apparatus (Pierce).

To extract stilbenoids secreted into the medium, the latter was partitioned three consecutive times with EtOAc in 500 mL separatory funnels. The ratio of medium to EtOAc was 5:3 (twice) and 5:2 (once). The combined EtOAc phases were collected in round bottles and evaporated to dryness under vacuum at 40 °C in a rotary evaporator (Büchi, Rotavapor R-200). Afterward, the extracts were dissolved in 1.5 mL of EtOAc, transferred to amber glass vials, and dried to completeness under a nitrogen stream using a Reacti-Vap III apparatus (Pierce). All extracts were kept at −20 °C until the necessary analyses were performed.

HPLC Analyses. For HPLC analysis, the dried extracts from media and roots were resuspended in 300 μL and 1.5 mL of methanol (MeOH), respectively. The samples were transferred to syringes and filtered through 0.2 μm nylon filters (Fisher) into glass inserts that were placed into 2 mL amber glass HPLC vials. Ten microliters of the filtrates was used for HPLC analyses. Standards and extracts were analyzed by reverse phase HPLC using a Dionex HPLC system coupled with photodiode array (PDA) and fluorescence detectors following the method developed by the Medina-Bolivar laboratory.[44] Concentration of stilbenoids in root and culture medium extracts was determined on the basis of the peak areas of the corresponding purified standards. Calibration curves for the quantification of stilbenoids were established using absorbance at 320 nm. A fluorescence detector (excitation at 335 nm and emission at 374 nm) was used for confirmation of the fluorescence properties of the stilbenoids. Reference compounds were the *trans* isomers of resveratrol (Biophysica), piceid (Biophysica), piceatannol (Axxora), and ε-viniferin (ACTIchem). Standard solutions for HPLC were prepared by dissolving the reference compounds in MeOH. Dilutions were done with MeOH to obtain concentrations from 5 to 80 ng/μL for resveratrol, from 5 to 40 ng/μL for piceid, from 0.8 to 40 ng/μL for piceatannol, and from 3 to 100 ng/μL for ε-viniferin.

Antioxidant Capacity of Selected Stilbenoids. The 2,2′-azinobis[3-ethylbenzthiazoline sulfonate] (ABTS) assay[45,46] was used to determine the antioxidant capacity of purified reference standards of the stilbenoids found in the medium and tissue of extracts from muscadine grape hairy roots. This assay was performed using the ABTS Antioxidant Assay Kit (Cayman Chemical) with modifications on 96-well plates. Standard samples were dissolved in DMSO (Sigma) instead of water, and the same volume of DMSO was added to the Trolox standard curve dilution wells ranging from 0 to 330 μM. Ten microliters of the assay buffer (solvent for Trolox) provided in the kit was added to the stilbenoid standard wells to account for the difference in volume. Stilbenoid concentrations ranged from 0 to 200 μM. The total volume in each well was 220 μL. Plates were covered with adhesive films and incubated on a shaker at room temperature for 5 min. The adhesive films were removed, and plates were read at 750 nm in a BIOTEK PowerWave XS spectrophotometer. Absorbance values for each stilbenoid were compared to the Trolox standard curve, and their antioxidant capacities were expressed as Trolox-equivalent (Trolox equiv) units.

Data obtained from standard curves of each purified stilbenoid using dilutions of 0, 25, 50, 75, 100, and 200 μM were used to calculate the ABTS free radical scavenging activity with the equation

$$\text{scavenging activity}\ (\%) = \frac{A_0 - A_i}{A_0} \times 100$$

where A_0 is the absorbance of the control sample (without purified stilbenoid standard) and A_i is the absorbance of the samples containing purified stilbenoid standard.

The effective concentration of each stilbenoid standard that is required to scavenge the ABTS radical by 50% (EC_{50}) was obtained by linear regression analysis of a dose−response curve plot between percentage of radical scavenging capacity and the molar concentration of each compound.

Statistical Analyses. Samples were assayed in triplicate, and the results are given as average ± SD. Data were analyzed by one-way analysis of variance (ANOVA), and Tukey pairwise comparison was performed when needed to confirm significant differences among treatment groups. Linear regression was performed for standard curves of stilbenoid standards. The statistical analyses were carried out using Minitab package version 16.1, and p values of <0.05 were considered to be significantly different.

RESULTS AND DISCUSSION

Establishment of Muscadine Hairy Root Cultures and Molecular Characterization. The infection of muscadine grape explants with *A. rhizogenes* strain ATCC 15834 produced several root initials (Figure 2b,c). Three lines per cultivar were selected according to growth vigor to continue the cultures in liquid BDS medium. Muscadine grape hairy roots showed a ropy phenotype (Figure 2d,e) and presented no root hairs as described for other species such as peanut (*Arachis hypogaea*) and *Trichosanthes kirilowii*.[24,47] This phenotype facilitated the

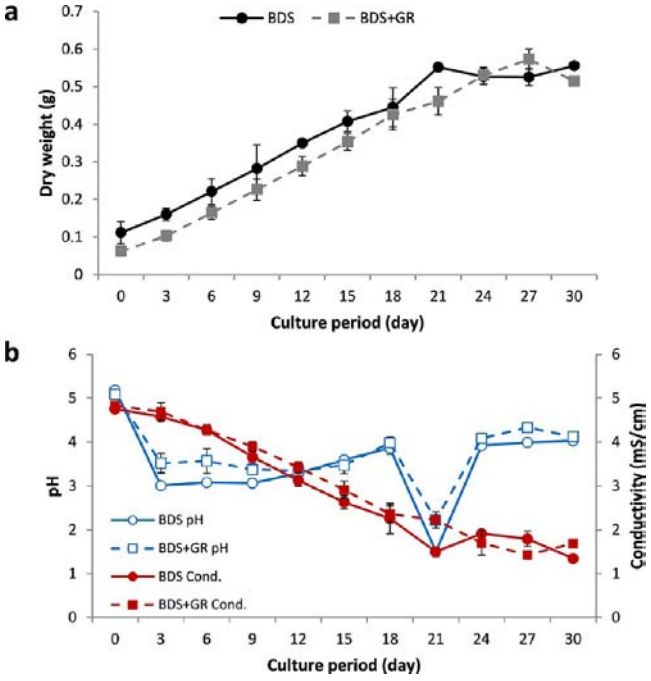

Figure 3. Growth of muscadine grape hairy root line Fry-3A in BDS and BDS+GR media: (a) growth kinetics in BDS and BDS+GR media; (b) values of pH and conductivity at different stages of growth. Each point represents the average of three biological replicates. Error bars represent standard deviations.

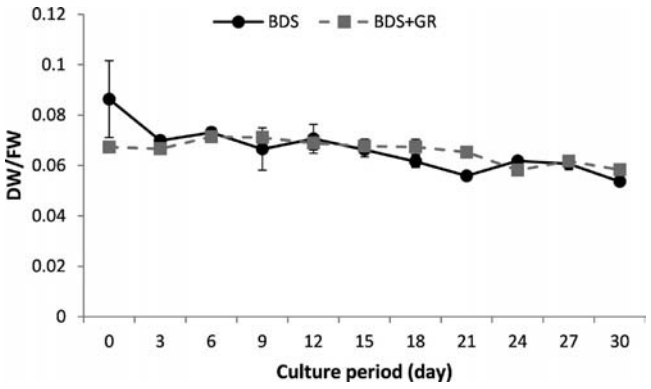

Figure 4. Dry weight to fresh weight ratio (DW/FW) of biomass of muscadine grape hairy root line Fry-3A in BDS and BDS+GR media. Values depict the average of three biological replicates, and error bars represent the standard deviations.

subculture of these hairy root lines into liquid cultures (Figure 2f). Root tips of 4−5 cm in length were better than smaller tips as inoculum. Additionally, the use of liquid medium containing plant growth regulators (IBA and BAP) increased secondary root formation but was not required for growth.

Extraction of nucleic acids to perform PCR for the insertion of the *rolC* and *aux1* genes in the plant genome proved to be challenging in muscadine grape hairy roots. Initially, we used commercially available DNA extraction kits and the TRIzol method with fresh and lyophilized tissue. However, the DNA yields were very low and the quality was poor, presumably due to the high concentration of phenolics and carbohydrates, as suggested from the 260/280 and 260/230 ratios from the NanoDrop spectrophotometer. To circumvent these problems, we used the CTAB method[43] with several modifications as described before. This method is longer and more laborious but allowed us to recover relatively high amounts of DNA from lyophilized tissue with good quality for downstream analysis.

PCR of lines Fry-3A (cv. Fry) and Nob-2Cot (cv. Noble) was used for confirmation of the integration of the two T-DNAs from the Ri plasmid of *A. rhizogenes* strain ATCC 15834 (pRi15834) into the muscadine grape genome. Because both T-DNAs, the left (T_L-DNA) and the right (T_R-DNA), have been described for agropine type *Agrobacterium* strains such as ATCC 15834, it was critical that our PCR analyses target a fragment of the *rolC* and *aux1* genes located on the independent T_L- and T_R-DNAs, respectively. The analysis showed that the *rolC* and *aux1* genes were present in line Fry-3A, confirming the integration of both T-DNAs (see the Supporting Information, Figure S1). Similar results were obtained with line Nob-2Cot (not shown). Moreover, PCR targeting of a segment of the *virD2* gene, which is located outside both of these T-DNAs, was negative. Therefore, the absence of any remaining *Agrobacterium* in the hairy root cultures was confirmed.

Growth Kinetics and Media Analyses. The type of medium and growth regulator supplements can have an important impact on tissue culture systems.[48] To test the effect of the supplementation with growth regulators (IBA and BAP) on growth performance, hairy roots of line Fry-3A were grown in BDS and BDS+GR media. During the 30 day culture we observed a different root morphology in these two media. Roots grown in BDS medium showed, on average, a diameter of 3 mm at age 21 days and two secondary roots every 5 cm. In contrast, hairy roots grown in BDS+GR medium showed more branching (4−5 branches every 5 cm) but were one-third thinner in diameter than those grown in BDS. The color of the roots was similar in both culture groups during the entire culture period, with the roots gradually turned from whitish to brownish as they aged. The roots of both groups also showed a colorless mucilage that tended to acquire a brownish color as the roots aged. Figure 3a compares the growth kinetics of the Fry-3A line in BDS and BDS+GR medium. No lag phase was observed under either media condition. A longer exponential growth phase was observed in roots cultured in BDS+GR medium (days 0−27) in comparison to BDS medium (days 0−24). It was also observed that the inoculation of root tips or segments of 4−5 cm length with two secondary roots routinely gave stable cultures with specific growth rates (μ) of 0.065 and 0.082 g/day for BDS and BDS+GR medium, respectively. The average doubling times (T_d) were 10.7 and 8.4 days for BDS and BDS+GR medium, respectively.

Prior to root inoculation, a decrease in the pH of the media from the original 5.8 to 5.58 and 5.59 for BDS and BDS+GR, respectively, was observed after autoclaving and could be caused by changes in the medium composition after autoclaving. When the growth started, the culture media presented no change in color and a transparent appearance. The pH dropped during the first 3 days and then remained relatively stable until day 9, when it started an increasing trend to pH 4 up to day 18 with a steep fall at day 21 and recovering at day 24 to the previous levels (Figure 3b). On the other hand, the conductivities of both types of media followed a decreasing trend as the growth progressed with values below 2 mS/cm at the stationary phase (Figure 3b).

The ratio of dry weight to fresh weight (DW/FW) was used by Kim and Park[49] as an index of the cell water content or cell size. In the present study, the DW/FW ratio of the hairy roots

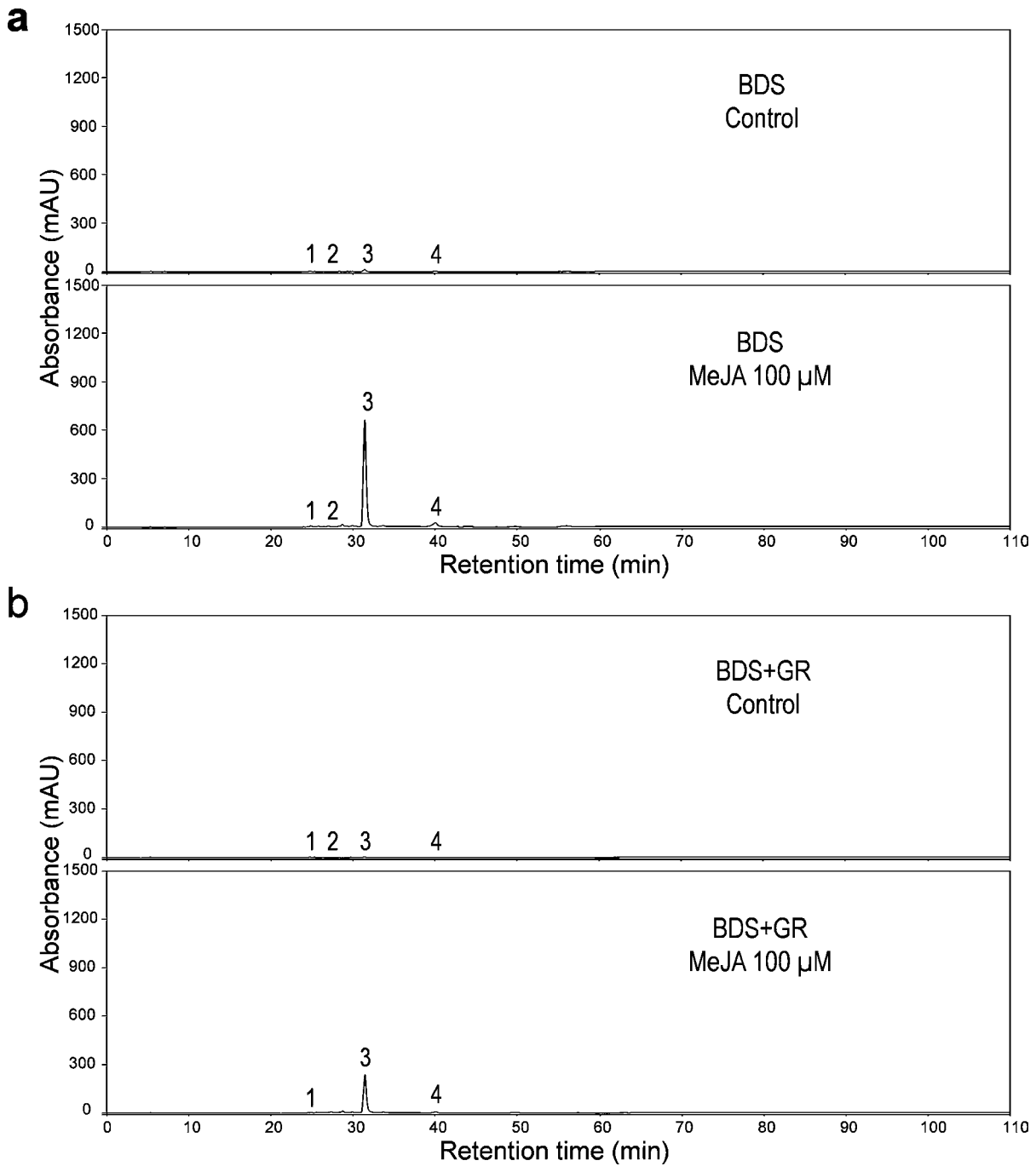

Figure 5. HPLC analysis of secreted stilbenoids in hairy root cultures of line Fry-3A upon treatment with MeJA for 24 h in (a) BDS and (b) BDS +GR media. HPLC chromatograms were obtained at 320 nm.

grown in either medium was relatively constant until day 24 for BDS and until day 27 for BDS+GR media, which were before the end of the exponential growth phase. The DW/FW ratio decreased thereafter as the roots were reaching the stationary phase (Figure 4), similar to what was observed in cell suspension cultures by Kim and Park.[49]

Effect of MeJA on Biosynthesis and Accumulation of Stilbenoids. Detailed studies on the induction of distinct stilbenoids in *V. rotundifolia* in vitro cultures have not been previously published. However, several studies have focused on the elicitation of these compounds in cell suspension cultures of related species such as *V. vinifera* and *V. amurensis*.[29,34,50] In this work we tested the effect of growth regulators on the growth performance and stilbenoid yield in hairy roots of muscadine grape treated with MeJA 100 μM. To do this, we used hairy roots from the lines Fry-3A and Nob-2Cot and analyzed the stilbenoids in extracts from tissue and medium by HPLC (see the Supporting Information, Figures S2 and S3). A recent study with hairy roots of muscadine grape reported the presence of resveratrol in these cultures. However, details for the establishment of muscadine grape hairy roots and quantification of resveratrol were not provided.[51]

Using our HPLC method we were able to identify four stilbenoids at different elution times: piceid (25 min), piceatannol (28 min), resveratrol (32 min), and ε-viniferin (40 min) in medium extracts. These compounds are labeled

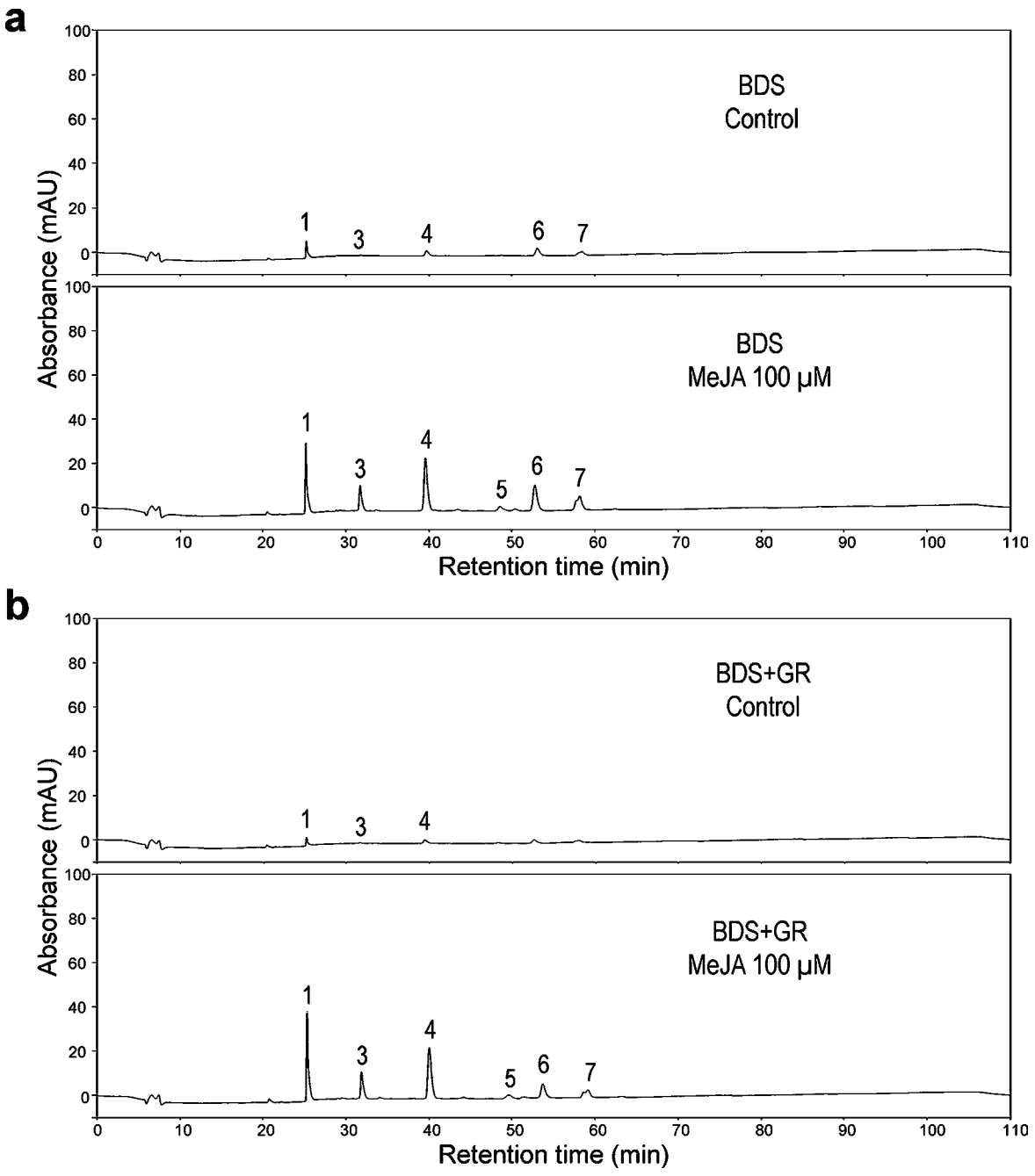

Figure 6. HPLC analysis of stilbenoids in the tissue of hairy root line Fry-3A upon treatment with MeJA for 24 h in (a) BDS and (b) BDS+GR media. HPLC chromatograms were obtained at 320 nm.

peaks 1, 2, 3, and 4, respectively, in the HPLC profiles (Figures 5 and 7). In root extracts, piceid, resveratrol, and ε-viniferin were detected (Figures 6 and 8). The observed elution times correlate with the polarity of compound given by their chemical structures. Three peaks corresponding to compounds with stilbenoid characteristics according to their UV spectra were also observed at different retention times in the tissue extracts: peaks 5, 6, and 7 at 49, 54, and 59 min, respectively (Figures 6 and 8). In general, we observed that the accumulation of resveratrol, piceid, and ε-viniferin was higher in the tissue than in the medium, whereas piceatannol was present mainly in the medium in both lines Fry-3A (Table 1) and Nob-2Cot (Table 2).

The levels of piceid were very low in medium extracts in Fry-3A with no difference between controls and elicited cultures. However, significant concentrations of this compound were found in the hairy roots extracts. We observed 2.2- and 3.5-fold increases in piceid with respect to the controls for the roots challenged with MeJA in BDS and BDS+GR media, respectively (Figure 9a). In the Nob-2Cot hairy root line, piceid was not detected in the media extracts but, similarly to the Fry-3A line, the concentrations in tissue yielded 4.1-fold (466.74 nmol/g DW in average) and 2.3-fold (159 nmol/g DW in average) increases when compared to the controls in BDS and BDS+GR media, respectively (Figure 10a). Stilbenoids are stored as glycosides in the cell,[52] which would explain why we have found these significant levels of piceid in the tissue

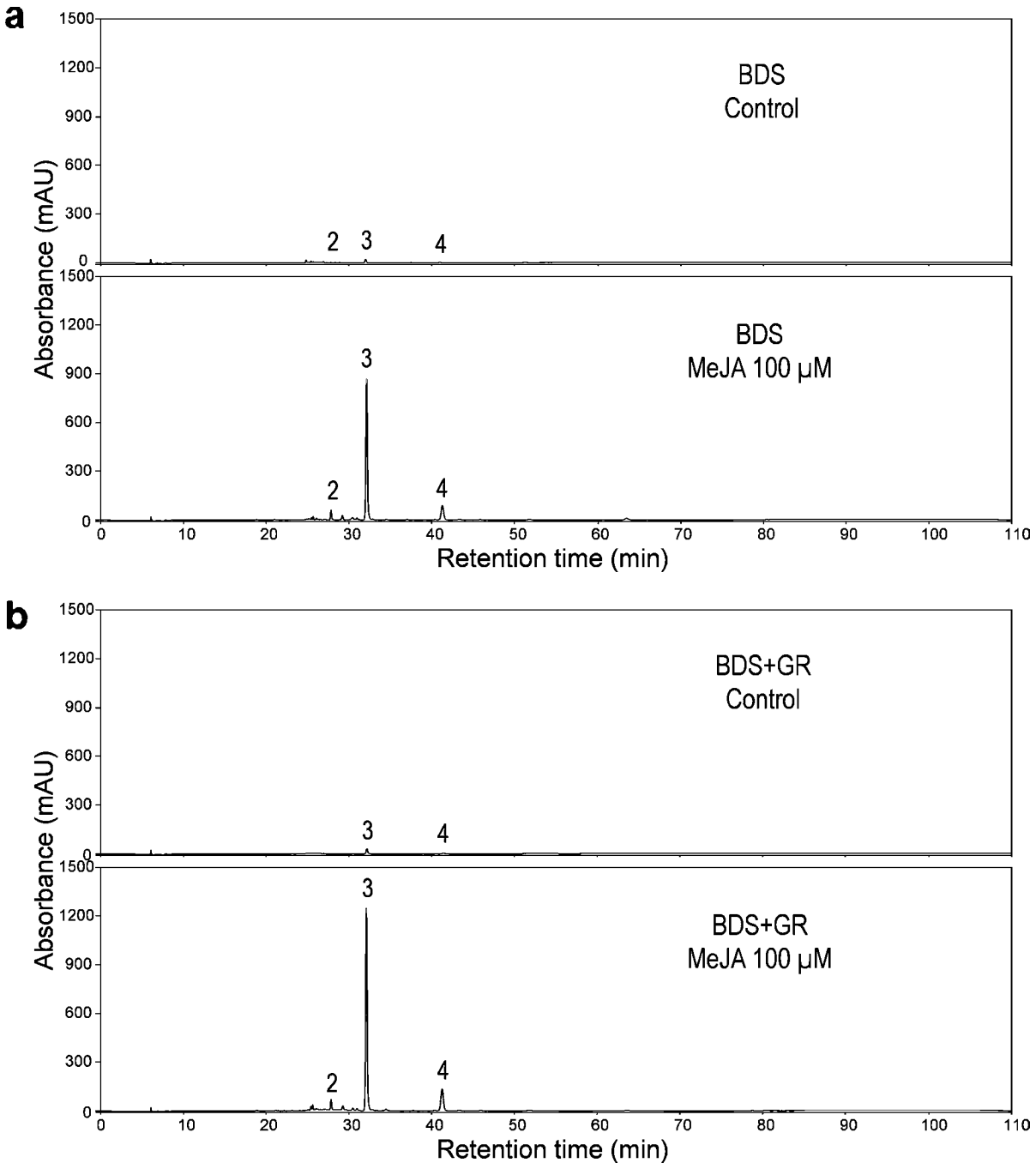

Figure 7. HPLC analysis of secreted stilbenoids in hairy root cultures of line Nob-2Cot upon treatment with MeJA for 24 h in (a) BDS and (b) BDS +GR media. HPLC chromatograms were obtained at 320 nm.

extracts. Previous research identified this compound as the main stilbenoid present in *Polygonum cuspidatum*, a medicinal species from Asia and a major commercial natural source for this compound.[53] Accordingly, studies done with *V. vinifera* cell suspension cultures treated with MeJA 20 μM have also found more accumulation of piceid in the cells than in medium.[34] In these cell suspension cultures the levels of piceid were 300 nmol/g FW. Similarly to what we observed in our hairy root system, basal levels of this compound were also observed in the control group of the cell cultures.

Unlike other stilbenoids, piceatannol concentrations in plants that produce this phenolic are very low. It has been reported to be induced in peanut calli exposed to UV,[54] in grapevine berries under ozone treatment,[17] and in cell suspension cultures treated with MeJA.[55] In our study, picetannol was not detected in the tissue of either of the hairy root lines. On the other hand, its levels in medium were below 10 nmol/g DW root in Fry-3A hairy roots grown in medium with and without growth regulators (Figure 9b). As for the Nob-2Cot line, traces of it were found in the medium of the root cultures treated with MeJA in BDS and BDS+GR media. In all cases these levels were <2 nmol/g DW hairy root tissue (Figure 10b). Nonetheless, we report here for the first time the observation of piceatannol in hairy roots of muscadine grape cvs. Fry and Noble.

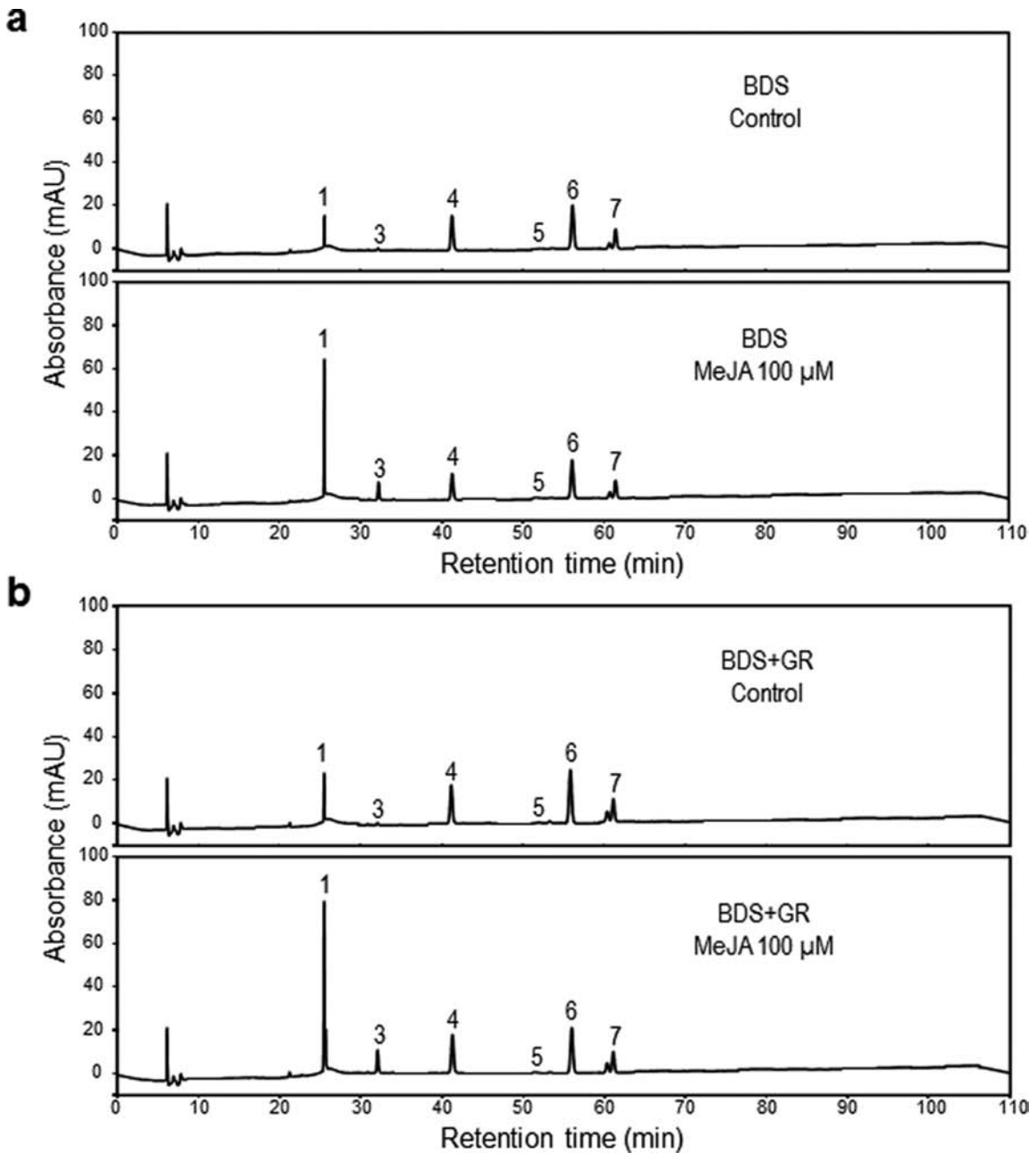

Figure 8. HPLC analysis of stilbenoids from the tissue of hairy root line Nob-2Cot upon treatment with MeJA for 24 h in (a) BDS and (b) BDS+GR media. HPLC chromatograms were obtained at 320 nm.

Table 1. Accumulation of Stilbenoids in Muscadine Grape Hairy Root Culture Line Fry-3A

	% ± SD; n = 3							
	piceid		piceatannol		resveratrol		ε-viniferin	
treatment	tissue	medium	tissue	medium	tissue	medium	tissue	medium
BDS								
control	99.91 ± 0.03	0.09 ± 0.03	nd[a]	100	98.42 ± 0.6	1.58 ± 0.6	99.58 ± 0.2	0.42 ± 0.2
MeJA 100 μM	99.96 ± 0.01	0.04 ± 0.01	nd	100	81.86 ± 5.3	18.14 ± 5.3	98.84 ± 0.2	1.16 ± 0.2
BDS+GR								
control	99.88 ± 0.1	0.12 ± 0.08	nd	100	98.94 ± 0.2	1.06 ± 0.2	99.5 ± 0.1	0.5 ± 0.1
MeJA 100 μM	99.96 ± 0.0	0.04 ± 0.00	nd	nd	90.22 ± 6.2	9.78 ± 0.9	99.39 ± 0.2	0.61 ± 0.2

[a]Not detected.

As in previous studies with cell suspension cultures where it has been observed that MeJA induces the synthesis and release of resveratrol into the medium,[33] in hairy roots of muscadine grape a similar response is observed. In the current study,

Table 2. Accumulation of Stilbenoids in Muscadine Grape Hairy Root Culture Line Nob-2Cot

	% ± SD; n = 3							
	piceid		piceatannol		resveratrol		ε-viniferin	
treatment	tissue	medium	tissue	medium	tissue	medium	tissue	medium
BDS								
control	100	nd[a]	nd	nd	97.04 ± 1.1	2.96 ± 1.1	99.80 ± 0.1	0.2 ± 0.1
MeJA 100 μM	100	nd	nd	100	87.67 ± 5.9	12.33 ± 5.9	98.04 ± 1.5	1.96 ± 1.5
BDS+GR								
control	100	nd	nd	nd	96.07 ± 1.2	3.93 ± 1.2	99.86 ± 0.04	0.14 ± 0.04
MeJA 100 μM	100	nd	nd	100	80.56 ± 14.4	19.44 ± 4.5	96.18 ± 1.2	3.82 ± 1.2

[a]Not detected.

resveratrol was the most abundant stilbenoid in medium extracts. However, when we compared medium versus tissue yields, we observed that most of it accumulated in the tissue. We found 28- and 19-fold increases of this stilbenoid with respect to their nonelicited controls in BDS and BDS+GR media in the line Fry-3A. Whereas the concentration of resveratrol in the root tissue was much higher than in the medium, the elicited roots showed a 2-fold increase in resveratrol when compared to the nonelicited controls on both media BDS and BDS+GR (Figure 9c). As observed in the Fry-3A line, resveratrol was the most abundant stilbenoid in the medium extracts of the Nob-2Cot line and also where the highest fold-change was observed. Thus, we found 32.8- and 39.5-fold increases with respect to the nonelicited control groups for the treatments with MeJA in BDS and BDS+GR media, respectively. In all cases, the detected amounts were <100 nmol/g DW tissue. In tissue extracts, the increase was on the order of 7- and 6.7-fold for the treatment groups when compared against their controls in BDS and BDS+GR media (Figure 10c).

ε-Viniferin is a dimer of resveratrol with strong anti-inflammatory properties and has been reported previously by Pezet et al.[56] in woody tissues from *V. vinifera* and recently in canes of *V. rotundifolia*.[57] Similarly, high endogenous ε-viniferin accumulation can be induced in muscadine grape as detected in the present study. We detected this compound in much higher concentrations in the tissue than in the media extracts. In the Fry-3A line we observed 3.4- and 5.3-fold increases in this resveratrol dimer than in the nonelicited controls for BDS and BDS+GR, respectively. On the other hand, the fold-increases were 9.3 and 6.7 in media extracts but with levels that were lower than 4 nmol/g DW (Figure 9d). ε-Viniferin was also found in significantly higher levels in the root tissue of the line Nob-2Cot, and the yields were 1.2- and 0.97-fold the levels found in the control groups for BDS and BDS+GR. In media, the increases were 12.5- and 27.3-fold the levels in the control groups for BDS and BDS+GR, respectively (Figure 10d). This compound was not detected in the elicitation studies by Belhadj et al.[34] using cell suspension cultures of *V. vinifera*; however, a more recent study on cell suspension cultures of a cross of *V. vinifera* Chasselas × *V. berlandieri* treated with MeJA showed induction of this dimeric stilbenoid in stirred bioreactors[55] and cell suspension cultures exposed to low-energy ultra sound in combination with MeJA.[58] We report here that for the first time the *trans* isomer of ε-viniferin has been identified in hairy root cultures of muscadine grape. The ability to produce and accumulate these biologically active compounds in hairy roots provides an alternative way to produce them because this plant material is suitable for extraction and purification with a relatively low volume of solvents.

The synthesis of specialized metabolites in hairy root cultures is often influenced by the incorporation of T-DNA genes in the host genome and the genome size or ploidy level of the cell.[59] Shkryl et al.[60] observed that the T-DNA *rol* gene loci (*rolA*, *rolB*, and *rolC*) played a major role in increasing anthraquinone production in transformed plant cells of *Rubia cardifolia*. Thus, we expect to find a similar correlation in the production of stilbenoids in muscadine grape hairy roots with respect to the *rol* genes inserted in their genome. Furthermore, studies performed by Kiselev et al.[29,30] and Dubrovina et al.[32] on transformed cells with *rol* genes and calli of *V. amurensis* found increased production of resveratrol. Future research will focus on the role that the *rol* genes exert on the synthesis of resveratrol and other stilbenoids in muscadine grape hairy roots.

Antioxidant Capacity of Muscadine Grape Stilbenoids. Free radicals play an important role in the autoxidation of unsaturated lipids in foods[61] as well as in oxidative cell damage in the human organism, resulting in a variety of pathological diseases.[62] Antioxidants such as the stilbenoid compounds are able to intercept the free radical chain of oxidations and to donate hydrogen from their phenolic groups, thereby forming stable free radicals that do not initiate or propagate further oxidation of lipids or other molecules.[63]

In this study, the scavenging antioxidant capacity of the four assayed stilbenoid standards was determined by the decrease in the absorbance of the ABTS radical at 750 nm induced by these antioxidant compounds. The analyses of these purified stilbenoids, which were also present in the muscadine grape hairy root extracts, showed that piceatannol was the strongest antioxidant compound followed by ε-viniferin, and resveratrol had a slightly higher scavenging activity than piceid when dissolved in DMSO (Figure 11a). The calculations of EC_{50}, which represent the amount of antioxidant needed to decrease the initial ABTS concentration by 50%, correlated with their radical scavenging activities. Thus, they showed, as expected, that piceatannol exhibited the lowest value (69 μM), followed by ε-viniferin (141 μM), then resveratrol (171 μM), and finally piceid, with an EC_{50} slightly higher than that of resveratrol (198 μM) (Figure 11b).

The synthesis of an immense variety of low molecular weight compounds or natural products with specialized functions is an inherent characteristic of plants. Several of these metabolites have defense properties in the plant and show beneficial effects to human health. Nonetheless, the capacity of plants to synthesize high levels of these compounds in a sustainable and reproducible manner is greatly affected by the physiological

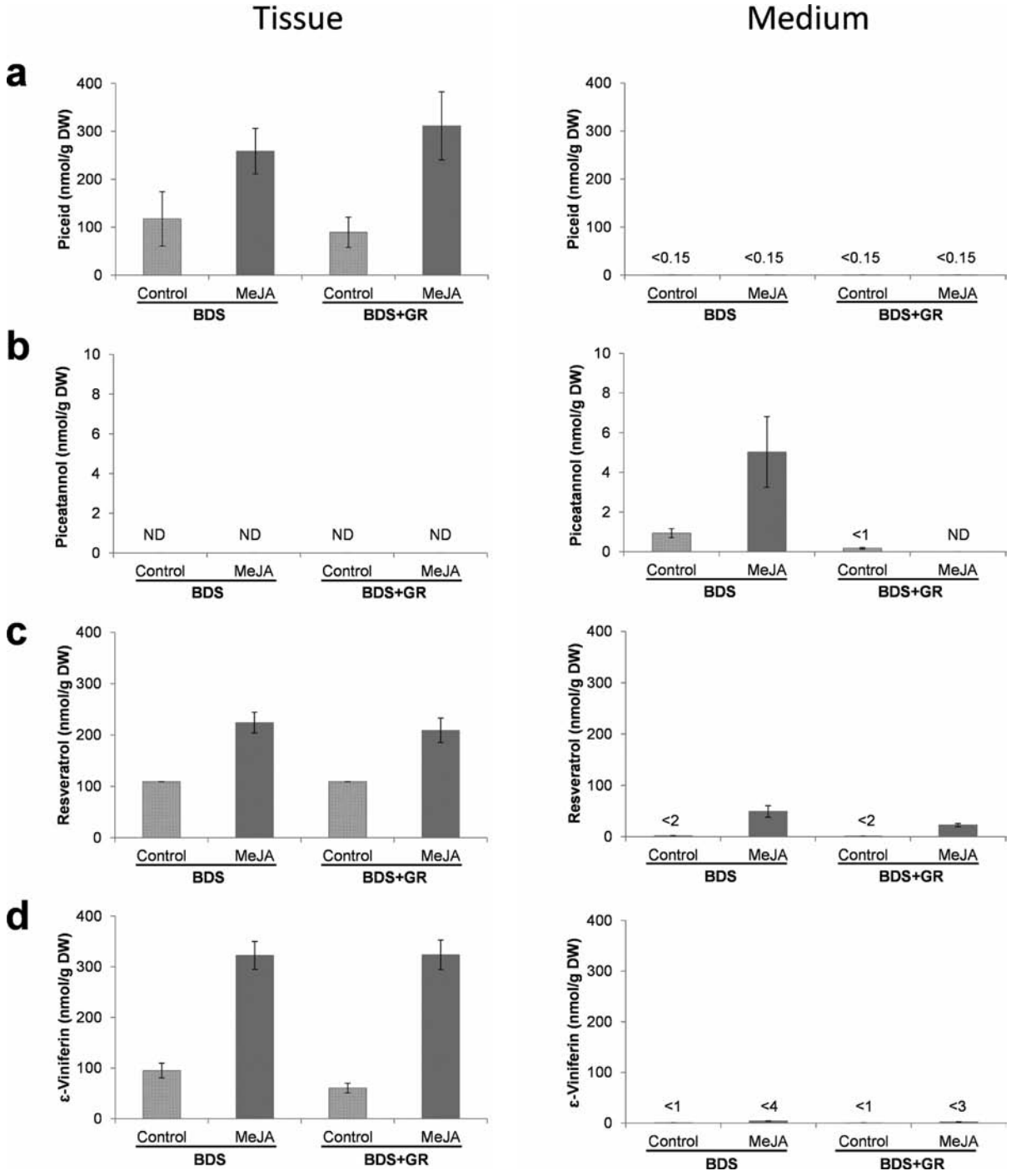

Figure 9. Accumulation of stilbenoids in medium and tissue of hairy root culture line Fry-3A upon treatment with MeJA for 24 h: (a) piceid; (b) piceatannol; (c) resveratrol; (d) ε-viniferin. Stilbenoids were quantified by HPLC, and each bar represents the mean ± SD of three biological replicates.

stage of the plant and environmental factors. Furthermore, many of the complex chemical structures exhibited by these natural products cannot be reproduced by chemical synthesis.

In this research we showed for the first time a detailed description on the establishment of muscadine grape hairy roots and the induction of the stilbenoids resveratrol, piceatannol, piceid, and ε-viniferin upon MeJA treatment. Unlike other stilbenoid-producing species, muscadine grape hairy roots accumulate resveratrol and other stilbenoids, with the exception of piceatannol, mostly into the root tissue; however, resveratrol is also secreted in lower levels into the culture medium along with traces of other stilbenoids. Additionally, the use of IBA and BAP did not significantly affect the biomass growth of these hairy root lines but induced morphological changes such as increased branching and decreased root diameter. On the other hand, the use of growth regulators did not affect the stilbenoid yields observed for hairy roots of muscadine grape grown in liquid BDS and BDS+GR media in combination with MeJA. Thus, a synergistic effect of MeJA and growth regulators in the biosynthesis of stilbenoids

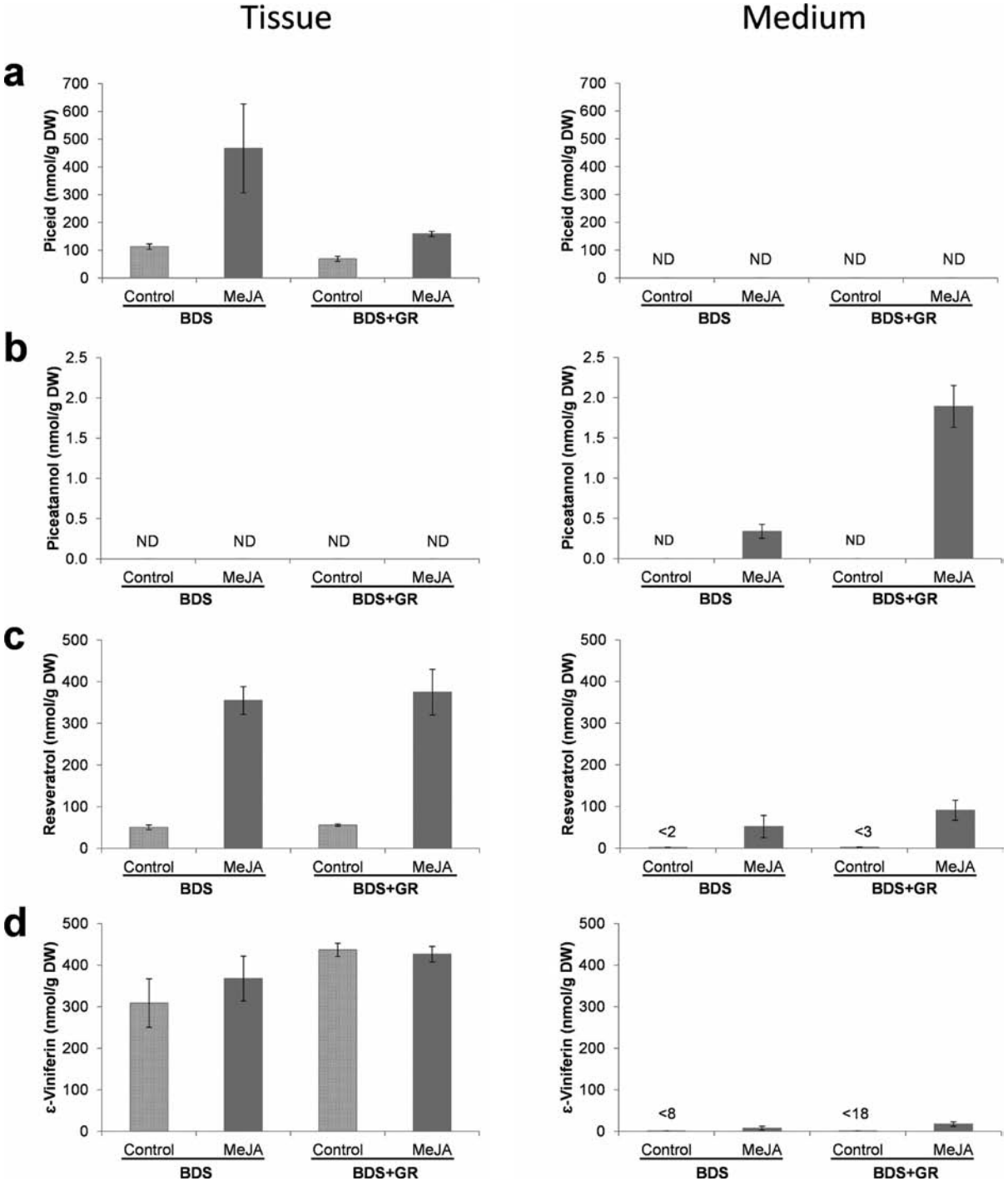

Figure 10. Accumulation of stilbenoids in medium and tissue of hairy root culture line Nob-2Cot upon treatment with MeJA for 24 h: (a) piceid; (b) piceatannol; (c) resveratrol; (d) *ε*-viniferin. Stilbenoids were quantified by HPLC, and each bar represents the mean ± SD of three biological replicates.

can be discarded. On the other hand, our study of the independent radical scavenging activity of muscadine stilbenoids showed significant low EC_{50} for piceatannol and *ε*-viniferin. These observations suggest that muscadine grape hairy root extracts containing resveratrol, piceid, *ε*-viniferin, and another three putative stilbenoids may exhibit higher radical scavenging activities through potential synergist mechanisms. Ongoing studies are focused on addressing the antioxidant activity of these extracts using the ABTS and an additional assay.

The muscadine grape hairy root culture systems provided the means to study the biosynthesis of stilbenoids as well as the factors that affect their regulation. Furthermore, muscadine hairy roots could be considered as an alternative and sustainable source of these important bioactive compounds. Finally, this system is potentially valuable in the study of the effect of the *rol* genes on the synthesis of resveratrol and its analogues as have been previously studied in calli and cell cultures.

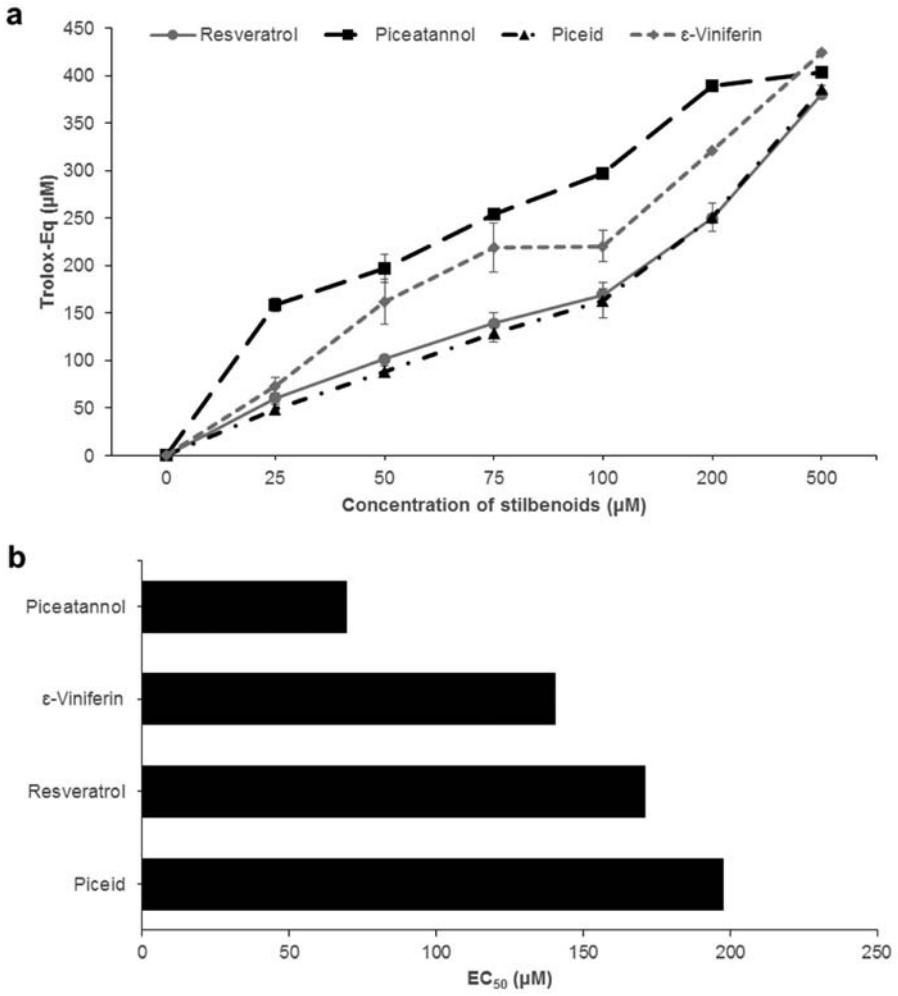

Figure 11. (a) Antioxidant capacity of selected stilbenoids found in muscadine grape hairy roots. (b) Effective concentrations (EC_{50}) required to scavenge the ABTS radical by stilbenoids present in muscadine grape hairy root cultures.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 reports the PCR analyses of hairy root line Fry-3A. Positive PCR result for the genes *aux1* and *rolC*. Additionally, a negative PCR result for the *virD2* gene shows the absence of the infecting bacteria in the hairy root tissue; **Figure S2** shows the treatment of muscadine grape hairy roots of line Fry-3A with MeJA. Controls (**a**) in BDS and (**c**) in BDS+GR media received ethanol (solvent for MeJA). Hairy roots in BDS (**b**) and BDS+GR (**d**) media were treated with MeJA (100 μM) for 24 h; and **Figure S3** shows the treatment of muscadine grape hairy roots of line Nob-2Cot with MeJA Controls (**a**) in BDS and (**c**) in BDS+GR media received ethanol (solvent for MeJA). Hairy roots in BDS (**b**) and BDS+GR (**d**) media were treated with MeJA (100 μM) for 24 h. This material is free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the National Science Foundation-EPSCoR (Grant EPS-0701890; Center for Plant-Powered Production-P3), Arkansas ASSET Initiative and the Arkansas Science and Technology Authority, and the Arkansas Biosciences Institute.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Special thanks to Dr. Maureen Dolan for assistance in obtaining muscadine grape seeds and to Dr. Gregory Phillips (Arkansas Biosciences Institute at Arkansas State University) for providing access to his laboratory to perform some aspects of this research.

■ DEDICATION

This paper is dedicated to the memory of John Hubstenberger, for his numerous contributions to plant tissue culture research.

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