Metabolomics driven analysis of *Erythrina lysistemon* cell suspension culture in response to methyl jasmonate elicitation

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**ABSTRACT**

An MS-based metabolomic approach was used to profile the secondary metabolite of the ornamental plant *Erythrina lysistemon* via ultra-performance liquid chromatography coupled to photodiode array detection and high resolution q-TOF mass spectrometry (UPLC-PDA-MS). Cultures maintained the capacity to produce *E. lysistemon* flavonoid subclasses with pterocarps amounting for the most abundant ones suggesting that it could provide a resource of such flavonoid subclass. In contrast, alkaloids, major constituents of *Erythrina* genus, were...
The genus *Erythrina* constitutes 115 species in the pea family “Fabaceae” which are distributed worldwide in tropical and subtropical regions growing as trees, often recognized in agriculture for their bright red flowers as coral or flame trees [1]. Alkaloids and phenolics are among the most widely distributed constituents in these flowering trees mostly localized in stem bark [2,3], roots [4] and seeds [5,6]. *Erythrina* alkaloids are tetracyclic spiroamines possessing an erythrinane skeleton. Over 90 *Erythrina* alkaloids have been isolated [7,8], often classified as dienoid or lactonic alkaloids. Interest in *Erythrina* alkaloids is mostly driven by its curare-like neuromuscular blocking effect. Moreover, *Erythrina* spp. possess a broad-spectrum of physiological activities such as anti-plasmodial activity due to the flavonoids and isoflavonoids [9], antioxidant and anti-inflammatory activities due to pterocarpans [10] and fungicidal activity associated with its alkaloidal content [11].

*Erythrina* genus has been extensively examined in terms of its taxonomy and chemical composition. However, very little information is available concerning biotechnological attempts for natural products production within that genus. Garcia-Mateos et al., showed that an unexpected profile of oxygenated alkaloids was observed in undifferentiated callus of *Erythrina Coralloides* and *Erythrina americana* [12]. Furthermore, San Miguel-Chavez et al., showed that jasmonic acid elicited *E. americana* cell culture has led to reduction in alkaloid accumulation [13]. Among the most common and effective elicitors used for stimulating secondary metabolites production in plant cell culture are the carbohydrate-like neuromuscular blocking effect. Moreover, *Erythrina* spp. possess a broad-spectrum of physiological activities such as anti-plasmodial activity due to the flavonoids and isoflavonoids [9], antioxidant and anti-inflammatory activities due to pterocarpans [10] and fungicidal activity associated with its alkaloidal content [11].

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1 mM L\(^{-1}\) MeJA. The dose 1 mM L\(^{-1}\) MeJA was previously optimized to elicit secondary metabolic pathways in cell cultures [17,18]. Furthermore, an increase in the concentration of MeJA resulted in retarded callus growth. The remaining flask was used as control by the addition of the same volume of sterile water. Cultures were kept at 23 °C ± 1 °C, with a 12 h photoperiod and maintained on a rotary shaker at 100 rpm. Cell culture samples were harvested at 0, 6, 12, 24 and 48 h post elicitation and kept at −80 °C until being analyzed.

**Extraction and UPLC-MS analysis of cell culture extracts**

Metabolites extraction followed the protocol developed for similar metabolite classes [18,19]. Briefly, lyophilized *E. lysistemon* cultures (20 ± 0.06 mg) were extracted with 1.8 mL aq.80% MeOH for 10 h using an orbital shaker in the dark. Extracts were centrifuged at 10,000g for 15 min and 1.4 mL of the supernatant was aliquot and evaporated under nitrogen till complete dryness. The dried residue was resuspended in 300 μL 45% aq. MeOH. For comparative analysis, the extracts were spiked with 2 μg umbelliferone as an internal standard (IS) and quantifications were determined from peak areas normalized based on the amount of recovered IS peak. The residue was re-suspended in 300 μL methanol and used for UPLC-MS analysis following the exact chromatographic conditions described by Farag et al. [20].

**Identification and quantification of metabolites and MS data multivariate analysis**

File Converter tool in X-Calibur software was used to convert UPLC–MS files to NetCDF file format and then further processed by AMDIS software for background subtraction and peak deconvolution. Metabolite identification was done via UV-VIS spectra (220–600 nm), retention times relative to external standards, mass spectra, and comparison to both the reference literature and phytochemical dictionary of natural product database. Quantification of alkaloids was calculated from the calibration curve of erythraline, pterocarpans using medicarpic standard, and for oleic acid using that of oleic acid standard detected using MS detector. Standard calibration curves were constructed for each standard using 4 concentrations spanning from 0.1, 1, 10 and 200 μg/mL. Assays were carried out in triplicate.

**MS data processing for multivariate analysis**

Relative quantification and comparison of metabolites profiles after UPLC-MS were performed using XCMS data analysis software, which can be downloaded for free as an R package from the Metlin Metabolite Database (http://137.131.20.83/download/) [21].

**Results and discussion**

**E. lysistemon cell culture metabolite profile**

Callus was produced from cut ends of scored *E. lysistemon* explants after 3 weeks. Chemical constituents of callus extracts were analyzed via UPLC/PDA/(−)ESI-qTOF-MS that allowed for the elution of cinnamates, flavonoids, alkaloids and fatty acids within 13 min (ca. 800 s). The elution order of secondary metabolites followed a sequence of decreasing polarity, whereby cinnamates and alkaloids eluted first, followed by flavonoid glycosides, free aglycones, prenylated aglycones and finally triterpenes and fatty acids. Simultaneously acquired UPLC–PDA and UPLC–MS total ion chromatograms of *E. lysistemon* cell culture extracts in positive and negative ionization mode are presented in Fig. 1. The identities, retention times, UV and MS spectral data observed for secondary metabolites are presented in Table 1 with a total of 53 identified metabolites. It is worth noting that this is the first comprehensive metabolic profile of *E. lysistemon* plant. Identified metabolites belonged to various classes (Table 1, Suppl. Fig. 1) including phenolic acids (cinnamates) i.e. *N*-caffeoyl aspartic acid (2), alkaloids *i.e.* erysotrine (6), pterocarpans *i.e.* isoneorautenol (30), isoflavonoids *i.e.* lysisteisoflavone (44), triterpenes *i.e.* oleic acid (53) and fatty acid *i.e.* hydroxy-9,11-octadecadienoic acid (45), with isoflavones and pterocarpans as the most abundant classes in cell culture extract. The structures of major metabolites identified in *E. lysistemon* and discussed throughout the manuscript are shown in Suppl. Fig. 1.

**Flavonoids**

Photodiode array detection provided an overview of the main flavonoid constituents (Fig. 1A). UV spectra (200–600 nm) were measured for flavonoid sub-classes including 12 isoflavones, 3 flavones, 4 isoflavonanes and 11 pterocarpans. Each sub-class exhibits a characteristic UV spectrum. For example, flavonones have a maximum absorbance near 265 nm with a second maximum between 320 and 340 nm (peak 9), whereas pterocarpans have λ max around 280–290 nm (42). Extracts were analyzed in positive and negative ion electrospray ionization (ESI) MS modes to provide a comprehensive overview of the metabolite composition. Compared to the positive-ion ESI mode (Fig. 1C), negative-ion MS spectra (Fig. 1B) revealed better sensitivity than in positive mode, especially in the elution range of flavonoids (200–500 s). In addition, negative-ion MS spectral characteristics showed strong [M − H\(^{+}\)]\(^{-}\) ions and lower chemical noise and consequently better sensitivity [22]. The positive ion ESI mass spectra were characterized by cations corresponding to [M + H\(^{+}\)]\(^{+}\), [M + Na\(^{+}\)]\(^{+}\) and fragment ions attributed to the sequential losses of isoprenyl (69 amu), malonyl (86 amu) and hexosyl (162 amu) groups. Few minor isoflavone peaks 13, 15, 18, 32 and 43 were only detected in positive ionization mode warranting the importance of acquiring data in both ionization modes. Two major flavone glycosides including dihydroxyflavone hexoside (m/z 415.102, [M − H\(^{+}\)]\(^{-}\) peak 9) and apiigenin hexosylmalonate (m/z 517.1702, [M − H\(^{+}\)]\(^{-}\) peak 11) were identified in cell culture. With regard to flavanone subclass, vogelin A (m/z 369.0999, [M − H\(^{+}\)]\(^{-}\) peak 25) and 5-deoxyglyasperin F/5-deoxylicoisoflavone (m/z 337.1085, [M − H\(^{+}\)]\(^{-}\) peak 27) exhibiting UV max around 310–320 nm typical for flavanones were measured.

**Pterocarpans**

Among flavonoid subclasses, pterocarpans amounted for the major forms in cell culture (11 peaks), exhibiting λ max around...
280–290 nm with isoneorautenol (m/z 321.1147, [M–H]– peak 30) as the most abundant (Table 1). Other identified pterocarpans include erythribyssin B (m/z 283.0598, [M – H]– peak 19), eryvarin D (m/z 335.1264, [M – H]– peak 20), dihydroisoneorautenol (m/z 323.1288, [M – H]– peak 31) and sandwicensin (m/z 337.1445, [M – H]– peak 42). The predominant loss of 69 amu (–C5H9, prenyl group) in the MSn spectrum of pterocarpans is diagnostic for the presence of the isoprenyl group; a total of 6 peaks showed this pattern. For example, erytragillin B (m/z 437.1993, [M – H]– peak 38) showed 2 mass fragments at m/z 368 and 299 indicative for 2 isoprenyl losses (–2 × 69 amu). The abundance of isoprenylated pterocarpan cell culture suggests for the presence of isoprenyl transferase enzyme with higher affinity toward pterocarpans. This is the first report for the accumulation of pterocarpans in *E. lysistemon* cell culture and suggests that it could provide a resource of that flavonoid subclass.

**Alkaloids**

With an increased sensitivity for detection of nitrogenous metabolites in positive mode, alkaloids could only be detected in that mode. Alkaloids that are known to predominate *E. lysistemon* plant extracts were almost absent in cell culture, except for few alkaloid peaks present at trace levels including erysotrine (m/z 314.1756 [M + H]+, peak 6), erythrartine/11-methoxyerysodine (m/z 330.1696, [M + H]+, peak 10) and erysotramidine (m/z 328.1534 [M + H]+, peak 12). In contrast, DOPA methyl ether (m/z 226.1073 [M + H]+, peak 8) was present as the major nitrogenous secondary metabolite identified in culture. No UV absorbance could be traced for alkaloid peaks, except for DOPA methyl ether showing distinct UV max at 270 nm. In tandem MS, alkaloids showed methyl losses from methoxy group (–15 Da).

**Phenolic acid (cinnamates)**

The most abundant nitrogenous compounds detected in cell culture were amino acyl hydroxycinnamic acid conjugates. A total of 5 peaks (2–5, 16) not previously reported in *E. lysistemon* plant tissue were identified in cell culture suggesting for an activation toward the production of acylcinnamates in cell culture. The predominant fragment of cinnamic acid derivatives in the MSn spectrum and characteristic UV max values at 298 and 325 nm are diagnostic for cinnamates; a total of 5 peaks showed similar UV (Table 1). MS/MS analysis confirmed the structure of N-p-coumaroylaspartic acid (3) m/z 278 and N-feruloylaspartic acid (5) m/z 308 from their respective product ions at m/z 163 and 193 indicative of a p-coumaroyl and feruloyl moieties, respectively, whereas N-cafeoylaspartic acid (2) gave a [M–H]– at m/z 294 with product ions m/z 132 for the aspartic acid moiety.

Differences in metabolites composition observed in *E. lysistemon* callus from its native plant are likely to be the result of genetic variation and/or lack of differentiation [22,23]. It is worth mentioning that there was no obvious qualitative or quantitative difference in the metabolite profile of the 2 different treatments of the calli (1 mg l−1 or 2 mg l−1 of Kinetin and 2,4D), results not shown.

**PCA of *E. lysistemon* MeJA elicited and control suspension culture observed in negative ionization mode**

Cell culture was further subjected to MeJA treatment to determine its impact on reprogramming of secondary metabolites as revealed via UPLC-MS analysis. To assess for changes in metabolite composition in response to elicitation as monitored via UPLC-MS traces of the different callus samples harvested at 0, 12 and 2 h post MeJA elicitation (Suppl. Fig. 2), principal
Table 1 Metabolites identified in *E. lysistemon* L. cell suspension methanol extract using UPLC–PDA–MS/MS in negative/positive ionization modes.

| Peak | rt (s) | UV (nm) | Metabolite | Class | Molecular ion m/z (±) | Error ppm | Composition | MS/MS |
|------|--------|---------|------------|-------|------------------------|-----------|-------------|--------|
| 1    | 122    | 285     | Dihydroxybenzoic acid pentosylhexose | Phenolic acid | 447.1139[M – H]⁻ | 1.2 | C14H20O13 | 378, 304 |
| 2    | 161    | 294, 325 | N-Caffeoylaspatic acid | Phenolic acid | 294.0592[M – H]⁻ | 9.1 | C13H17NO7 | 175, 132 |
| 3    | 198    | 294, 326 | N-p-Coumaroylaspartic acid | Phenolic acid | 278.0661[M + H]⁺ | 3.2 | C13H16O7 | 163, 132 |
| 4    | 209    | 287, 312 | N-(Hydroxycinnamoyl) tyraminehexose | Phenolic acid | 476.1877[M + H]⁺ | 9.3 | C24H24O8 | 314 |
| 5    | 220    | 294, 325 | N-Feruloylaspatic acid | Phenolic acid | 308.0758[M – H]⁻ | 5.9 | C14H20O7 | 193, 132 |
| 6    | 237    | 282     | Erysootrine | Alkaloid | 314.1756[M + H]⁺ | –1.6 | C19H20N2O4 | 280 |
| 7    | 238    | 272, 340 | Apigeninpentosyl hexose | Flavone | 563.1423[M – H]⁻ | –3 | C20H20O14 | 269, 253 |
| 8    | 238    | 280     | DOPA methyl ether | Alkaloid | 226.1073[M + H]⁺ | 0.6 | C11H15O4 | 178 |
| 9    | 245    | 270, 332 | Dihydroxyflavone hexose | Flavone | 415.1012[M – H]⁻ | 3.5 | C17H16O4 | 253 |
| 10   | 252    | 325     | Erypthrinol/11-Methoxyerysodine | Alkaloid | 330.1696[M + H]⁺ | 1.3 | C19H20N2O4 | 312, 280 |
| 11   | 262    | nd      | Apigeninhexosylmalonate | Flavone | 517.1702[M – H]⁻ | 4.0 | C24H24O8 | 269, 253 |
| 12   | 263    | nd      | Erysootramidine | Alkaloid | 328.1534[M – H]⁻ | 2.9 | C19H20O7 | 313 |
| 13   | 277    | 282, 286 | Demethylmedicarpin hexosylmalonate | Pterocarpan | 503.1158[M + H]⁺ | 5.1 | C24H24O12 | 255 |
| 14   | 278    | 280, 308 | Diacctoxy benzoic acid | – | 237.0397[M – H]⁻ | 3.3 | C11H14O4 | 215, 174 |
| 15   | 289    | 282, 286 | Demethylmedicarpin hexosylmalonate | Pterocarpan | 503.1162[M + H]⁺ | 5.1 | C24H24O12 | 255 |
| 16   | 292    | 272, 319 | N-Cinnamoyl-Asparatic acid | Isolavone | 262.0717[M – H]⁻ | 1.5 | C19H20N2O5 | 218, 146 |
| 17   | 310    | 262, 308 | Dihydroxyisoflavone | Isolavone | 253.0497[M – H]⁻ | 3.5 | C13H16O5 | 194 |
| 18   | 310    | 282, 286 | Dihydroxyisoflavone | Pterocarpan | 255.0637[M + H]⁺ | 5.7 | C13H16O5 | 174 |
| 19   | 337    | 232, 285 | Erythribysin B | Pterocarpan | 283.0598[M – H]⁻ | 4.9 | C16H16O4 | 269, 253, 214 |
| 20   | 349    | 280, 335 | Eryarin D | Pterocarpan | 335.1264[M – H]⁻ | 7.3 | C21H16O4 | 271, 266, 241 |
| 21   | 358    | 284     | Unknown isoflavone | Isolavone | 355.1173[M – H]⁻ | 4.0 | C20H16O5 | 333, 267 |
| 22   | 395    | 280     | Unknown isoflavone | Isolavone | 369.1324[M – H]⁻ | 5.4 | C21H16O6 | 321 |
| 23   | 408    | 280, 335 | Apigenin | Flavone | 269.0448[M – H]⁻ | 2.9 | C17H16O4 | 178 |
| 24   | 485    | 280, 310 | Unknown isoflavone | Isolavone | 397.1288[M – H]⁻ | 1.3 | C22H24O6 | 353 |
| 25   | 485    | 230, 287 | Vogelin A | Isolavone | 369.0999[M – H]⁻ | 5.2 | C20H16O5 | 329, 269 |
| 26   | 507    | nd      | Oleamonic acid trihexose | Triterpene | 943.5253[M + H]⁺ | –5.4 | C48H54O18 | 457 |
| 27   | 510    | 287, 307 | 5-Decoxyglycacerin | Isolavone | 337.1085[M – H]⁻ | –1 | C20H20O6 |  |
PCA of E. lysistemon MeJA elicited and control suspension culture observed in positive ionization mode

To provide more overview on the effects of elicitation on E. lysistemon cell culture metabolome, samples were also analyzed in positive ionization MS condition. PCA score plots derived from MS peaks in positive ionization mode were comparable to those in negative mode concerning segregation of samples at 0 h from 12 and 24 h. The PCA model (Fig. 3A) explained 93% of the total variance in the first component, PC1, whereas the second principal component, PC2 presented 6% of the variance. Although comparable score plots in PCA were derived from both data sets, loading plots revealed a slightly different set of metabolites contributing for sample clustering. As revealed in Fig. 3B, the major group that stood out in this plot corresponded to MS signals for dimethoxy-isoflavone (43), isoeorautenol (30) and an unknown pterocarp found more enriched in unelicited cell culture samples harvested at 0 h. In contrast, negative loading plot results along PC1 revealed that the triterpene glycoside “oleanolic acid tri-hexoside” (26) and an unknown sterol (49) (Fig. 3B) levels were higher in the MeJA treated samples and accounting for its segregation at 12 h and 24 h from 0 h time point. The enrichment of the major pterocarp “isoeorautenol” (30) in the untreated control cell culture samples (Fig. 3B) concurs results derived from negative ionization mode and highlighting the negative impact of MeJA on pterocarps biosynthetic branch. The decrease in pterocarpan levels in response to MeJA treatment is contrary to previous reports in Medicago truncatula cell culture [17] and lupines [26], suggesting that a differential response to MeJA exists in various legume species. This is the first report of MeJA differential effect on terpenoid accumulation versus pterocarps in E. lysistemon cell culture (Fig. 4). Studies focused on the genetic bases of MeJA
**Fig. 3** UPLC-qTOF-positive ionization MS (m/z 100–1000) principal component analyses of *E. lysistemmon* unelicited cell culture samples (○), cell cultures treated with 1.0 mM MeJA at 0 h (○), 12 h (△) and 24 h (+) (*n* = 3). The metabolome clusters are located at the distinct positions in two-dimensional space prescribed by two vectors of principal component 1 (PC1 = 93%) and principal component 2 (PC2 = 6%). (A) Score Plot of PC1 versus PC2 scores. (B) Loading plot for PC1 and PC2 contributing to mass peaks and their assignments, with each metabolite denoted by its mass/rt (s) pair.

**Fig. 4** Diagram showing major secondary metabolite pathways with represented major structures that are up regulated in *E. lysistemmon* plant, cell culture and in response to MeJA elicitation.
elicitation will help affirm induction hypothesis derived via metabolite profiling. It should be noted that oleanolic acid tri-hexoside conjugate was not detected by visual examination of unelicited cell culture chromatograms, suggesting that coupling of metabolomics for analysis of elicited samples presents a powerful methodology for identification of novel metabolites. Quantification of the major differential metabolites in elicited cell culture is presented in Table 2.

Conclusions

This study provides the first report on *E. lysistemon* cell suspension culture metabolite fingerprint via UPLC-MS. A metabolomic approach was used to investigate secondary metabolites viz. alkaloids, flavonoids and triterpenes and their reprogramming in response to MeJA elicitation. The results confirm MeJA elicitation effect on terpenoid accumulation and extend our knowledge base concerning secondary metabolism in other legume species [27]. Comparative metabolic profiling of *E. lysistemon* cell suspension culture and in response to elicitation using MeJA, revealed an activation in sterol/triterpenes formation, see model depicted in Fig. 4. The effect of other elicitors on secondary metabolites accumulation in *Erythrina* cell culture could also provide more holistic insight into elicitation effect within that genus and how it can reprogram its different secondary metabolite pathways.

Conflict of Interest

The authors declare that they have no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jare.2016.07.002.

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| Metabolites (µg g⁻¹) | *E. lysistemon* cell suspension |
|---------------------|---------------------------------|
|                     | 0 h | 12 h | 24 h |
| Erystronine        | 2.8 ± 0.9 | 3.4 ± 2.1 | 0.6 ± 0.5 |
| Erystronamide      | 10.8 ± 3.8 | 8.9 ± 2.5 | 6.0 ± 1.4 |
| ErylysinA          | 36.0 ± 8.2 | 22.2 ± 5.4 | 12.7 ± 2.6 |
| Sandwicensin       | 10.7 ± 2.9 | 3.4 ± 0.5 | 2.2 ± 0.5 |
| Oleanonic acid     | 406 ± 32.1 | 4907 ± 163 | 4838 ± 237 |
| Erythrabissint     | 1268 ± 85 | 268 ± 59 | 243 ± 18 |
| Isonoractenol      | 2217 ± 89 | 473 ± 16 | 564 ± 47 |
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