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Acetogenins and alkaloids during the initial development of *Annona muricata* L. (Annonaceae)

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Abstract: In plants, the presence and distribution of specialized metabolites during the early stages of development are not documented enough, even though their biosynthesis is one of the most important strategies for survival. In this study, five alkaloids and four acetogenins were detected in *Annona muricata* L. during early development seedling, including three phases of root emergence and three of seedling formation. Hexane and alkaloid extracts were obtained from each organ, which were analyzed in a gas-mass chromatograph and in a high-performance liquid chromatograph coupled with a photodiode array UV detector (HPLC-DAD). This research shows the presence of the acetogenins *cis*-uvarimicin IV, mosinone, muricina B, and *cis*-annonacin-10-one, as well as of the alkaloids reticuline, coreximine, anonaine, asimilobine, and nornuciferine, both groups with a variable organ-specific distribution, related with the formation of organs and tissues.

Keywords: Annonaceae; germination; secondary metabolites; seedlings.

1 Introduction

Seed germination and seedling formation are marked by the metabolic activation that triggers the set of gradual and progressive changes in size, structure, and function. The plants invest the resources, endowed by the mother plant, in the form of reserve material, to accomplish the mechanisms that allow them to develop and establish themselves in the ecosystem [1–3]. Among this mechanisms are the division, expansion, and cellular differentiation that result from the differential expression of the genetic material. When the plant becomes an autotrophic organism, the metabolic costs are supported by photosynthesis [1].

During this initial process, plants are exposed to multiple biotic and abiotic factors that lead to death, making it a critical phase of their life. At this stage, so vulnerable to predation and environmental changes, plants activate mechanisms for their defense and survival [4], which may be physical (such as the development of pubescences or thorns) or chemical (such as the production of specialized metabolites). These are dependent on the increase of the biomass, on the reserve material stored in the seeds and, when the plant grows, on the photosynthesis.

In contrast to the morphological, physiological, and genetic aspects of germination and seedling development, knowledge about metabolites specialized in defense or protection is less documented. However, its biosynthesis is one of the most important strategies for its survival, and it has been analyzed especially in studies on herbivory [5]. Research on metabolites specialized in early development has reported around 200 molecules, including alkaloids, cyanogenic glycosides, anthocyanins, flavonoids, and simple phenols [6].

The presence and organ-specific distribution of specialized metabolites during these stages is variable among species. This distribution can be part of different strategies such as the defense of roots against insects, microorganisms, and parasites of the soil [7], or of leaves and stems against herbivores and pathogens, or as protection against UV radiation [8], among others.

Among plants, the Annonaceae family is characterized by having a specialized active metabolism, with privileged biosynthetic routes for the production of alkaloids

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and “annonaceous acetogenin” molecules. Both are toxic substances and have diverse biological activities [9–14]. A few species have reported the presence of more than 934 benzyloquinoline alkaloids [15–17] and more than 540 acetogenins [18]. These metabolites have been isolated from different organs and tissues of adult plants, and as far as it has been possible to deduce, the two metabolic pathways are expressed during all the stages of life, but the diversity of its metabolites is possible to find in different organs and moments. As for the alkaloids, organ-specific variation during early development has only been described in two studies, referring to the oxoaporphin alkaloid liriodenine. De-la-Cruz and González Esquinca reported [19] that the variation of liriodenine in *Annona diversifolia* is associated with the degree of development and differentiation of tissues, so this alkaloid was first detected in the endosperm of the seeds at 5 and 10 days of imbibition, later in the radicle and the hypocotyl, reaching its maximum concentration in six-leaved seedlings (2827.1 nmol g⁻¹ dry matter), but was not found in cotyledons and leaves. These data allow us to infer and prove that the alkaloid forms part of the antifungal defense system on *Rhizopus stolonifer* (Ehrenb: Fr.) and *Aspergillus glaucus* L. fungi isolated from the same seeds and which disappear just as the radicle emerges [20].

From *Annona muricata* L., a popular species in traditional medicine and widely studied, have been isolated 99 acetogenins [21] and 10 alkaloids of fruits, leaves, roots, and seeds [15, 16, 22–24]. With the exception of the acetogenins rolliniastatin-2 and laherradurin [25], the moment in which the biosynthesis of these compounds is initiated and its location or variation in the plant is unknown. Understanding the presence and distribution of alkaloids and acetogenins during the early stages of the plant development contributes to the understanding of the dynamics of specialized metabolism, as well as it provides some elements to guide research on the defense mechanisms in which these specialized metabolites participate. Therefore, this study had as an objective to determine the distribution pattern and its dependence to stages of early development of four acetogenins and five benzyloquinolinic alkaloids, constitutive compounds of *A. muricata*.

2 Materials and methods

2.1 Stages of growth evaluated

Two phenological stages were analyzed: root emergence (phases 1, 2, and 5 cm of radicle) and the formation of

seedlings (leaf primordium phases (Fp), two leaves and six leaves). A total of six development phases were analyzed, which, in order to establish analogies with other phenological works, were quadrated to stages 0 and 1 according to the BBCH scale [26] (Table 1).

In order to obtain the plants in different stages of their development, the germination of 1000 seeds was induced using the technique of “between paper” [27], in rolls of brown paper with 50 seeds, which were placed in plastic containers of 1 L with 150 mL of water. The seeds were washed every third day to prevent the appearance of fungi, and changes of paper and water were also made. Then, 200 seeds were harvested in the root emergence stage, while another 500 seeds were kept in paper rolls until reaching the Fp stage. After reaching this stage, the seeds were sowed in 500-mL extruded polystyrene foam cups, with previously sterilized soil, in an autoclave FE-397 (Felisa, Jalisco, México) until reaching the two- (2 Lf) and six-leaf (6 Lf) phases. The whole experiment was maintained inside a Conviron® series CMP4030 germination chamber (Conviron, Winnipeg, Canada) with a controlled environment at 28 °C, with a 12-h photoperiod, fluorescence lamps at an intensity of 500 µM m⁻² s⁻¹, and a relative humidity between 65% and 75%. From each stage of development, radicles, roots, stems, and leaves were carefully separated with pruning shears. Each organ was placed in brown paper bags and made to dry at room temperature for 4 days.

2.2 Obtaining acetogenic and alkaloidal extracts

To obtain the acetogenin profiles, 2 g of dry plant material was used per phase. An extraction with hexane J.T. Baker was made, with a reactive grade, at a continuous reflux, in a Soxhlet equipment, for 8 h, three times. The extract was concentrated under reduced pressure in a rotavaporator Caframo VV2000 and stored in glass jars until its high-performance liquid chromatograph (HPLC) analysis. The remaining vegetal material from the hexane extraction was performed with an alkaloidal extraction by the acid-base method [28]. After thorough grinding, samples were moistened with a saturated Na₂CO₃ solution and left to dry for 48 h at room temperature. Alkaloids were extracted with CHCl₃ by constant stirring for 1 h, and then filtered and washed with distilled water. The CHCl₃ phases were extracted into a 1-M HCl solution before being alkalized to pH 9.5 with a saturated solution of Na₂CO₃. They were then re-extracted with CHCl₃ and dried with anhydrous Na₂SO₄, filtered, and evaporated at about 25 °C to obtain

Table 1: Morphophysiological characteristics of germination and seedling development of *A. muricata*.

Stages	Escale BBCH	Growth phases (days)	Morphophysiological events	Keys
Germination 	05	1 cm of radicle (12–14)	Mobilization of substances of endosperm reserves, elongation of radicle	Rd1
	06	2 cm of radicle (15–17)	Mobilization of substances of endosperm reserves, elongation of radicle	Rd2
	07	5 cm of radicle (20–22)	Differentiation of the embryonic axis in radicle and hypocotyl. Mobilization of substances from the endosperm reserves	Rd5
Seedling 	11	With foliar primordium (25–28)	Partially open green cotyledons. First pair of true leaves and separated from the apex of the stem. Branched root. Photosynthesis	Fp
	12	With two leaves (30–35)	First pair of leaves in their final size. Independence of the seedling. Photosynthesis	2 Lf
	16	With six leaves (60–64)	Six dark green leaves, developed root system. Vegetative growth. Photosynthesis	6 Lf

Stages and phases growth were adjusted to BBCH scale (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie).

total alkaloids. All reagents and disolvents were supplied by JT Baker, San Pedro Xalostoc, Edo de México, México.

2.3 Acetogenin determination

To determine the identity of the acetogenins, both the UV absorption spectrum and the organ from which it was isolated in other works with *A. muricata* were considered (Table 2).

The method using a high-performance liquid chromatograph HPLC coupled with a photodiode array UV detector (HPLC-DAD) (Perkin Elmer Flexar, Norwalk, CT, USA) in reverse phase (Spheri-5 RP-18, 250×4.6 mm i.d., 5-μm particle diameters (Perkin Elmer Instruments). The mobile phase consisted of acetonitrile-methanol-water

(J.T. Baker) (80:10:10 isocratic) [32]. The flow rate was 1 mL min⁻¹, and the temperature of the column compartment was held at 30 °C. UV detection at 220 nm was used. The analyzed sample volume was 20 μL at 1 mg mL⁻¹ dissolved in methanol.

2.4 Determination of alkaloids

The determination of the alkaloids was carried out by the method employed by Egydio et al. [33] with some modifications under the following conditions: a volume of 1 μL of the samples dissolved in methanol at 5 mg mL⁻¹ was analyzed in a GC/MS Perkin Elmer Clarus Model 680 GC (Waltham, MA, USA), coupled to a Clarus SQ8T MS spectrometer (Waltham, MA, USA), with a 1:20 split. The capillary column used as a stationary phase was a Perkin Elmer Elite-1 (32 m×0.32 mm and 0.25-mm sheet thickness). Helium was used as the carrier gas at a flow of 1.2 mL min⁻¹. The column temperature conditions were initially at 150 °C for 1 min, with an increase of 10 °C min⁻¹–280 °C and maintained at that grade for 16 min. The temperature of the injector was 300 °C. Also, mass fragmentation was collected at 70 ev with 2.89 scans s⁻¹ and the fragments were detected from 50 to 500 Da. The temperature of the ion source and that of the quadrupole was 270 °C.

Table 2: UV absorption wavelength (maximum peak) of acetogenins detected from *A. muricata*.

Acetogenin	Peak maximum UV absorption	Tissue	Reference
Muricin B	210	Leaf	[29]
<i>cis</i> -annonacin-10-one	209	Seeds	[30]
Mosinone	202	Stem bark	[31]
<i>cis</i> -uvariamicin IV	221.3	Roots	[31]

2.5 Data analysis

The results of each molecule detected during germination and the seedling stage ($n=3$) are expressed in units of absorbance per gram of dry plant material ($\mu\text{A g}^{-1}\text{ mv}$). To determine the accumulation dynamics of each molecule during the development of the seedlings, the relative abundance was calculated, taking into account the area of the chromatograms. Relative abundance = [(amount detected at $\times$ time/highest amount detected in a particular tissue) $\times 100$].

2.6 Statistical analysis

To compare the differences in the concentration of alkaloids and acetogenins in the stages of development evaluated, the nonparametric Kruskal-Wallis test was performed, and to separate between treatments, a post-hoc Mann-Whitney test was made. Variations of the molecules during the development with polynomial regressions were performed in the Past program (Natural History Museum, University of Oslo, Oslo, Norway). Differences were considered significant at a level of $p \leq 0.05$.

3 Results

During the two stages of growth, it was possible to detect the presence of four acetogenins and five alkaloids with variable organ distribution (Figure 1).

3.1 Acetogenins and alkaloids in roots

In the roots, two acetogenins were detected from the early stages of growth: *cis*-uvarimicin IV ($p=9.104 \times 10^{-5}$, $\chi^2=25.955$) when the radicle just emerges (Rd1), and mosinone ($p=3.918 \times 10^{-5}$, $\chi^2=27.839$), 25 days later, when the Fp has been reached. The most abundant acetogenin in the development phase of Fp is mosinone, while *cis*-uvarimicin IV is in the 2 Lf. In this tissue, both molecules persist during the 2 Lf to 6 Lf (Figure 2A).

Five alkaloids were found (Table 3), namely, anonaine, nornuciferine, and reticuline from early stages, at the beginning of germination, when the radicle reaches 1 cm in length (Rd1). On the other hand, the asimilobine ($p=4.192 \times 10^{-5}$, $\chi^2=27.686$) alkaloid appears when the radicle measures 5 cm (Rd5); and coreximine (3.65×10^{-5} , $\chi^2=27.99$) at 30 days, when the seedling has reached the Fp. In the roots these compounds increase in relation to

the development of the plant, being more abundant in the 2 Lf seedling, with the exception of asimilobine and anonaine ($p=0.000112$, $\chi^2=25.507$), which remains constant during leaf formation (Figure 2B).

3.2 Acetogenins and alkaloids in stems

In the stems as in the roots, the presence of the acetogenins *cis*-uvarimicin IV ($p=0.008929$, $\chi^2=9.42$) and mosinone ($p=0.002964$, $\chi^2=11.58$) was determined. These appear for the first time in the foliar Fp and both increase in the 6 Lf seedling, with *cis*-uvarimicin IV being more abundant than mosinone in the two stages (Figure 2C).

Four alkaloids were identified, two of them nornuciferine ($p=0.001867$, $\chi^2=12.567$) and reticuline ($p=0.001495$, $\chi^2=13.011$) are found in the stem of seedlings with primordium leaf, while anonaine ($p=0.0001495$, $\chi^2=13.011$) and asimilobine ($p=0.001278$, $\chi^2=13.325$) appear only in seedlings with six leaves. With the exception of asimilobine, very abundant in the stems of seedlings in the 6 Lf, the other alkaloids show a similar amount throughout the development. Unlike the roots, in this organ, coreximine was not detected (Figure 2D).

3.3 Acetogenins and alkaloids in leaves

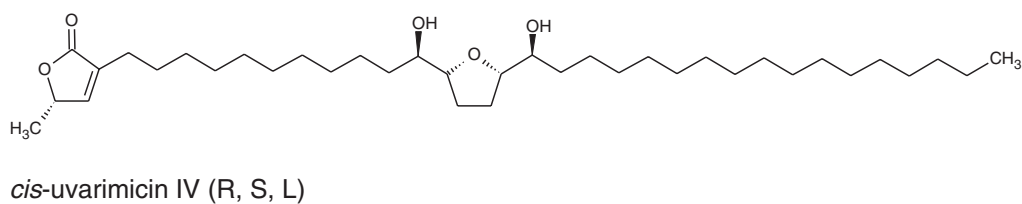
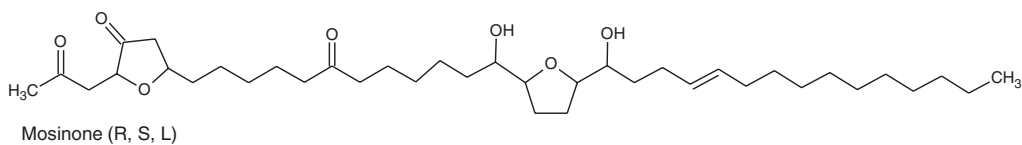
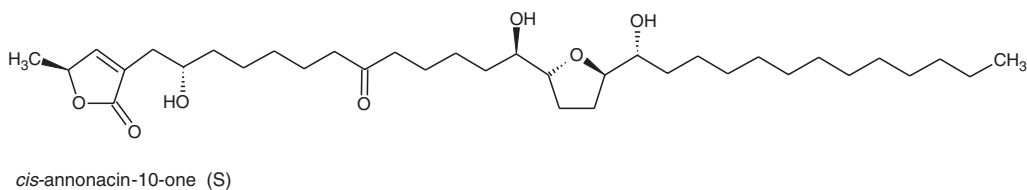
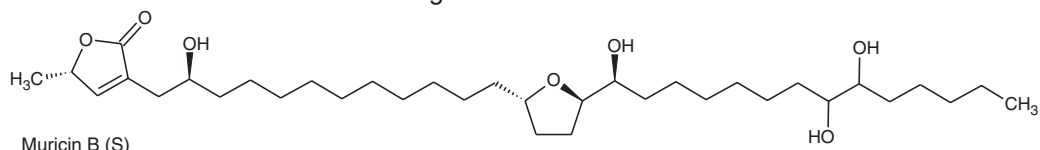
In the leaves, the presence of *cis*-anonacin-10-one ($p=0.001867$, $\chi^2=12.57$), mosinone ($p=0.003624$, $\chi^2=11.18$), and muricin B ($p=0.001888$, $\chi^2=12.5$) was determined; two of them were not detected in roots and stems (muricin B and *cis*-anonacin-10-one). The three acetogenins appear during the Fp. *cis*-anonacin-10-one increases its concentration as the plant develops; the opposite occurs with muricin B, whose content decreases as the plant develops. Mosinone exhibits a more erratic behavior (Figure 2E).

Four alkaloids were found in leaf seedlings: anonaine ($p=0.001495$, $\chi^2=13.011$) and reticuline ($p=0.01495$, $\chi^2=13.011$) were detected in 2 Lf seedlings, while asimilobine ($p=0.001278$, $\chi^2=13.325$) and coreximine ($p=0.001278$, $\chi^2=13.25$) were found in six seedlings after 5 days. The levels of these alkaloids in the plant increase in relation to its development (Figure 2F).

3.4 Accumulation dynamics

Acetogenins and alkaloids in *A. muricata* occur in different organs and at different times (accumulation dynamics). The richness (number) and abundance of alkaloids

Acetogenins



Alkaloids

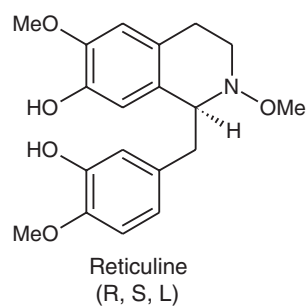
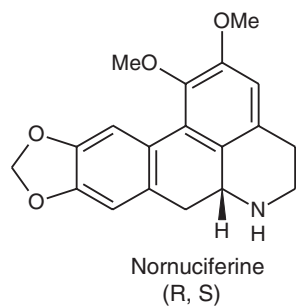
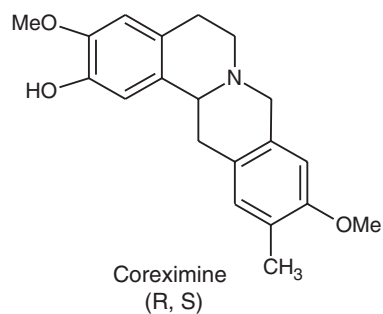
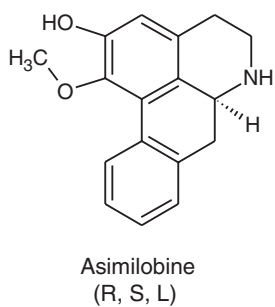
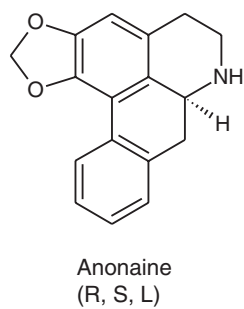


Figure 1: Acetogenins and alkaloids detected in the developmental stages of *A. muricata* seedlings. Their presence in roots (R), stems (S), and leaves (L) are indicated.

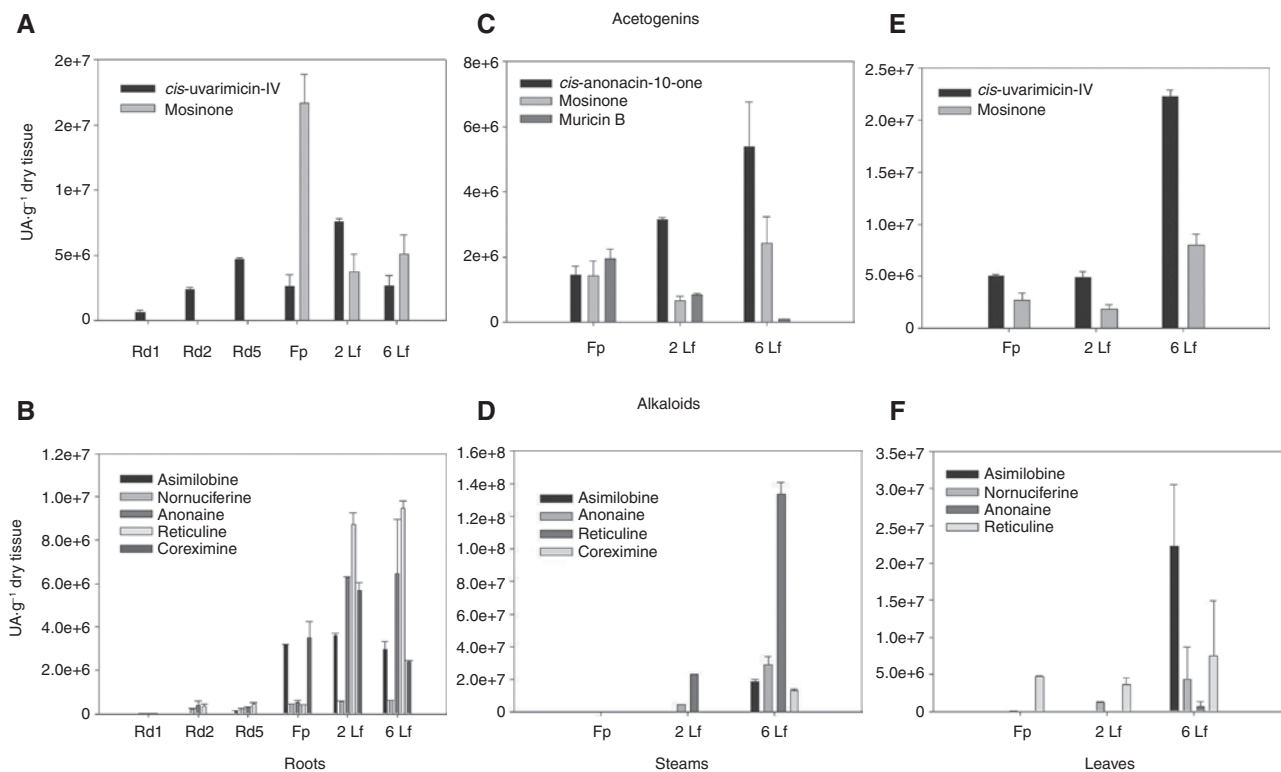


Figure 2: Ontogenetic variation and organ-specific distribution of acetogenins and alkaloids during the early development of *A. muricata*. Roots (A, B); stems (C, D); leaves (E, F). The values represent the average of three replicates, and standard deviations are indicated. The difference between means was considered significant at 95% confidence interval.

Table 3: Mass spectrometry data for the five identified alkaloids from *Annona muricata*.

Alkaloid	EI-MS M/Z (relative intensity, %)
Anonaine	265, 264 (100), 207 (53.6), 102 (34.48)
Asimilobine	267 (43.14), 266 (100), 252 (36.25), 250 (22.13), 236 (15.48)
Coreximine	327 (29.28), 207 (27.54), 178 (100), 176 (33.61), 151 (24.12), 150 (71.67)
Nornuciferine	281 (43.29), 280 (100), 250 (19.79), 221 (14.94)
Reticuline	329, 192 (100), 207 (16.62), 193 (13.37), 177 (27.32)

increase as the plant develops, with the highest concentration being observed during the 6 Lf. In contrast, there is no similar pattern in acetogenins: while their abundance is the same in the 6 Lf, each molecule has a unique dynamic (Figure 3).

4 Discussion

Different morphophysiological events occur during the initial phases of *A. muricata* development (Table 1). After

imbibition and during the metabolism's activation, the reservoirs are mobilized to start elongation and embryo differentiation, the aperture of the testa, and the emergence of the radicle. At the same time, the specialized metabolism (secondary metabolism) is expressed, a fact which is documented in 99 species of different families [6]. Important metabolic investments are also destined within the Annonaceae, especially for alkaloid and acetogenin biosynthesis, clearly established within this family, as is documented in multiple systematized reports by Cavé et al. [34] and Lucio et al. [17], among others.

The ontogenic relation between the specialized metabolism and the development of the plant in Annonaceae has been documented in *A. diversifolia* Saff. (synonym of *Annona macrophyllata* Donn. Sm), by De la Cruz and González-Esquinca [19], alongside the biosynthesis of liriodenine during the seed's imbibition. Also, in a paper by Brechú-Franco et al. [35], it was demonstrated that idioblasts (specialized cells that contain alkaloids and acetogenins) are formed during the development of the radicle and the hypocotyl.

In the present study, it was found that the biosynthesis of both alkaloids and acetogenins in *A. muricata* occurs simultaneously during the initial stages of development.

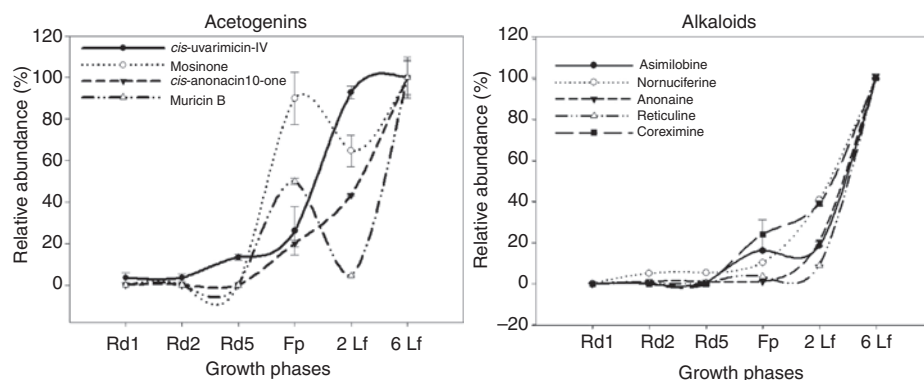


Figure 3: Comparison of relative abundances (accumulation dynamics) of acetogenins and alkaloids during six phases of initial development of *A. muricata*. The relative abundances were calculated: $(\text{amount detected at } \times \text{time} / \text{highest amount detected in a particular tissue}) \times 100$.

Both types of molecules appear in variable proportions, developmental stages, and organs, signaling their crucial role in the plant development. The documented acetogenins had been found before in the stems, roots, leaves, and seeds of adult specimens [18, 32, 34, 36–48], but it was not until now that their presence was detected in the early stages of development.

Four monotetrahydrofuran acetogenins were detected in the early stages. These include *cis-uvarimicin IV*, *mosinone*, *muricina B*, and *cis-annonacin-10-one*; all of them related with organ differentiation. *cis-uvarimicin IV* and *mosinone* appear in the roots when growth still depends on reserve material, with only one report of its presence in the roots of an adult specimen [49], while *muricin B* and *cis-annonacin-10-one* are only present when the plant is photosynthetically active. Both acetogenins can be found also in the leaves and seeds of adult plants [50], a trait that could indicate their dependence on photosynthesis. The presence of these acetogenins in the plant's photosynthetic phases, indicate a kind of survival function that occurs in the vulnerable stages of development. Also, it was noted that the acetogenins *laherradurin* and *rolliniastatin-2* were present in *A. muricata* seedlings [25], where it serves a larvicide function. González-Coloma et al. [51] reported that *laherradurin* is more active than *rolliniastatin-2* on *Spodoptera littoralis* Boisduval, a folivore, with an 86% and 29.7% mortality rate, respectively. The biosynthesis of Annonaceous acetogenins is not known, but with some methods, such as the isolation of precursors, as well as the presence of numerous asymmetric centers and biomimetic hemisyntheses, it is thought that acetogenins originated from a precursor of three carbon atoms and a fatty acid [34]. The latter is very abundant in the seeds of the species of the Annonaceae family.

The insecticidal activity of *cis-annonacin-10-one*, in *A. muricata* has only been demonstrated on *Oncopeltus*

fasciatus Dallas and on *Spodoptera frugiperda* Smith and Abbo [52, 53] or fungicidal properties, as shown by *scuamocin*, *scuamocin G*, and *scuamostatin*, on *Phytophthora infestans* Mont [54]. These acetogenins have not been found in *A. muricata* before or in the present analysis; nonetheless, the presence of these molecules must suppose an evolutionary advantage, which enables the plants to adapt to their environment.

The following alkaloids were detected in the early developmental stages of *A. muricata*: *reticuline*, *coreximine*, *anonaine*, *asimilobine*, and *nornuciferine* (Figure 1). These have also been found in the organs of other adult plants. These alkaloids, in a similar manner to the acetogenins, show a spatial-temporary and organ-specific distribution.

Reticuline serves as an intermediary alkaloid within the diversification in the biosynthetic pathway that produces six benzyloquinoline alkaloid synthesis [55]. It has been found in the roots, stems, and leaves of adult *A. muricata* specimens [15, 16, 22–24], although their presence in their seeds are undocumented. In the present study, *reticuline* was detected in radicle emergence, specifically, in roots of over 1 cm in length (Rd1), which signals an early biosynthesis that depends on the endosperm's reserved material. However, *reticuline* is also present all over the seedling, during its early phase, and its biosynthesis depends on photosynthetic activity. In both cases, a considerable energy cost is produced, which is to be expected from an *ad hoc* biosynthetic machinery, whose presence repeats frequently in these and other specialized metabolites.

Anonaine, an aporphynic alkaloid, is the most found alkaloid in plants of the Annonaceae family after *liriodenine* [17]. There is no evidence of its presence in *A. muricata* seeds. Thus, *anonaine*, and its biosynthesis, occurs in roots during the early development stage.

Furthermore, it is possible to detect anonaine on leaves when these start to develop, in the same way as liriode-nine, produced during imbibition and emergence of the radical, while both are located in the seedlings, leaves, roots, hypocotyls, and stems of *A. diversifolia* [19]. The fact that anonaine can be found only in the stems of the six-leaved seedlings allow us to infer the lack of transport from the roots, as well as the biosynthetic machinery that might be present in the leaves. Anonaine has important fungicidal properties against dermatophyte fungus and bacteria [56].

Asimilobine, another aporphine, and the third most reported alkaloid in the specimens from the Annonaceae family [17], is found in roots (that have reached 5 cm in length), also in leaves, when the seedling has two and six leaves, and has been reported in fruits also [22, 57].

Additionally, the presence of normuciferine in the fruits [22, 57], alongside its early detection, enables us to infer that it could be (at least initially) a contribution from the mother plant, in contrast with the other alkaloids.

Finally, coreximine, a protoberberine alkaloid, appears to be dependent on the photosynthetic activity of the plant, as it is only found in the leaves' development stages [23, 58]. The same result is also found in adult *A. muricata* specimens. For the biosynthesis of this type of alkaloids, a major number of enzymes seems to be involved than in the biosynthesis of aporphinics [24].

Pathway diversification in the biosynthesis of specialized metabolites and its distribution along the seedling enable us to infer that the plant invests more resources in the protection of life-dependent organs, such as the leaves due to their photosynthetic function, or the roots with their important role in water and nutriment transportation [5]. Therefore, roots and leaves would have a bigger production and accumulation of alkaloids and acetogenins. Implicated within the mechanisms of biosynthetic regulation, it is possible to find genes, enzymes, cells, tissues, and organelles, all of which depend on a process linked to organelle and tissue differentiation. Accordingly, the seedling posits resources from distinct reservoirs for its development and simultaneously for specialized metabolite biosynthesis. Acetogenin and alkaloid variations throughout development is characteristic of the specialized metabolism's dynamics, modulated by gene expression, which, in turn, is different for every molecule (Figures 7 and 8). As it has been demonstrated in seedlings of *Arabidopsis thaliana* (L.) Heynh [6, 59–62]. This species produces anthocyanins after 3–5 days of sprouting; likewise, in *Catharanthus roseus* (L.) G. Don, catharanthine, tabersonine, cindoline, and vincristine alkaloids accumulate after 7–10 days of sprouting [6, 63–65].

The neurotoxic activity found in four of the five detected alkaloids, as well the cytotoxic and pesticide activity of the acetogenins [51], enables us to suppose that these compounds are produced and maintained all along the complete development of the plant, as part of the defense systems against distinct pathogen species. Paired with the aforementioned process, at least in the case of alkaloids, there is, during early development, a diversification of the biosynthetic pathway beginning with reticuline [66], its main intermediary, which leads to protoberberine or aporfinoid biosynthesis. Although, to date, the biosynthetic pathway of alkaloids of species of the Annonaceae family is not known, there is no in vivo interconversion between them.

The increment of some alkaloids is related with some phyto regulators, for example, the exogenous addition of gibberellic acid (GA_3) specifically in morphine's yield [67]. Likewise, IAA's metabolism is tightly related with the specialized metabolism in some species, as is the case of plants in the order Capparales, which include *Arabidopsis*, where indolic glucosinolates and other indolic defense compounds (camalexine) are formed, starting from the biosynthetic pathway of indol acetic acid [68]. This suggests a relation between gibberellins' and auxines' expression, formed during division, elongation, and differentiation of cells alongside [67] the development of roots and leaves and the expression of the specialized metabolism.

5 Conclusion

During its first steps' stages of development, *A. muricata* expresses the biosynthesis of diverse acetogenins and alkaloids. This fact is related among the multiple biosynthetic processes that are linked alongside the formation of tissues and organs. To be sure, the importance of the expression for the plant is manifested the biosynthesis capable of initiating and being preserved from the sprouting until (at least) the seedling stage. In some cases, this synthesis depends on the apparition of leaves or can even be organ specific. Even though the understanding of these processes still appears partial and fragmentary (being related mostly within defense systems), the type of studies, such as the present, can be directed toward the research of the importance the processes have for the plants that produce them.

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References

- Bewley JD, Black M. Seeds. Physiology of development and germination, 2nd ed. New York: Springer, 1994.
- Pritchard SL, Charlton WL, Baker A, Graham IA. Germination and storage reserve mobilization are regulated independently in *Arabidopsis*. Plant J 2002;31:639–47.
- Bewley JD, Bradford K, Hilhorst H, Nonogaki H. Seeds physiology of development, germination and dormancy, 3rd ed. New York: Springer, 2013.
- Vivanco JM, Cosío E, Loyola-Vargas VM, Flores HE. Mecanismos químicos de defensa en las plantas. Investig Cienc 2005;341:68–75.
- Kaplan I, Halitschke R, Kessler A, Sardanelli S, Denno RF. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. Ecology 2008;89:392–406.
- De la Cruz Chacón I, Riley-Saldaña CA, González-Esquinca AR. Secondary metabolites during early development plant. Phytochemistry Rev 2013;12:47–64.
- Baetz U. Root exudates as integral part of belowground plant defence. Chapter 3. In: Belowground defence strategies in plants. Cham, Switzerland: Springer, 2016: 45–67.
- Mierziak J, Kostyn K, Kulma A. Flavonoids as important molecules of plant interactions with the environment. Molecules 2014;19:16240–65.
- Pereira MN, Justino AB, Martins MM, Peixoto LG, Vilela DD, Santos PS, et al. Stephalagine, an alkaloid with pancreatic lipase inhibitory activity isolated from the fruit peel of *Annona crassiflora* Mart. Ind Crops Prod 2017;97:324–9.
- Muganza DM, Fruth B, Nzunzu JL, Tuentner E, Foubert K, Cos P, et al. *In vitro* antiprotozoal activity and cytotoxicity of extracts and isolated constituents from *Greenwayodendron suaveolens*. J Ethnopharmacol 2016;193:510–6.
- Meira CS, Guimarães ET, Macedo TS, da Silva TB, Menezes LR, Costa EV, et al. Chemical composition of essential oils from *Annona vepretorum* Mart. and *Annona squamosa* L.(Annonaceae) leaves and their antimalarial and trypanocidal activities. J Essent Oil Res 2015;27:160–8.
- Liaw CC, Liou JR, Wu TY, Chang FR, Wu YC. Acetogenins from Annonaceae. In: Kinghorn AD, Falk H, Gibbons S, Kobayashi J, editors. Progress in the chemistry of organic natural products. New York: Springer International Publishing, 2016.
- Prabhakaran K, Ramasamy G, Doraisamy U, Mannu J, Rajamani MJ. Polyketide natural products, acetogenins from graviola (*Annona muricata* L), its biochemical, cytotoxic activity and various analyses through computational and bio-programming methods. Curr Pharm Des 2016;22:5204–10.
- Rakotomanga M, Razakantoanina V, Raynaud S, Loiseau PM, Hocquemiller R, Jaureguierry, G. Antiplasmodial activity of acetogenins and inhibitory effect on *Plasmodium falciparum* adenylate translocase. J Chemother 2004;16:350–6.
- Leboeuf M, Cavé A, Bhaumik PK, Mukherjee BR. The phytochemistry of the Annonaceae. Phytochemistry 1982;21:2783–813.
- Cavé AM, Leboeuf M, Waterman PG. The aporphinoid alkaloids of the Annonaceae. Chapter 4. In: Pelletier SW, editor. Alkaloids: chemical and biological perspectives, New York, USA: Wiley, 1987:133–270.
- Lúcio AS, da Silva Almeida JR, da-Cunha EV, Tavares JF, Barbosa Filho JM. Chapter alkaloids of the Annonaceae: occurrence and a compilation of their biological activities. Alkaloids Chem Biol 2016;74:233–409.
- Bermejo A, Figadère B, Zafra-Polo MC, Barrachina I, Estornell E, Cortes D. Acetogenins from Annonaceae: recent progress in isolation, synthesis y mechanisms of action. J Nat Prod 2005;22:269–303.
- De la Cruz Chacón I, González-Esquinca AR. Liriodenine alkaloid in *Annona diversifolia* during early development. N Prod Res 2012;26:42–9.
- De la Cruz Chacón I, González-Esquinca AR, Guevara Fefer P, Jiménez García LF. Liriodenine, early antimicrobial defense in *Annona diversifolia*. Z Naturforsch C Biosci 2011;66c:377–84.
- Soheil ZM, Mehran F, Sonia N, Gokula M, Hapipah MA, Habsah AK. *Annona muricata*(Annonaceae): a review of its traditional uses, isolated acetogenins and biological activities. Int J Mol Sci 2015;16:15625–58.
- Hasrat J, Bruyne TD, Backer JP, Vauquelin G, Vlietinck A. Isoquinoline derivatives isolated from the fruit of *Annona muricata* as 5-HT₂ 5-HT_{1A} receptor agonists in rats: unexploited antidepressive (lead) products. J Pharm Pharmacol 1997;49:1145–9.
- Lannuzel A, Michel PP, Caparros-Lefebvre D, Abaul J, Hocquemiller R, Ruberg M. Toxicity of Annonaceae for dopaminergic neurons: potential role in atypical parkinsonism in Guadeloupe. Mov Disord 2002;17:84–90.
- Kotake Y, Okuda K, Kamizono M, Matsumoto N, Tanahashi T, Hara H, et al. Detection and determination of reticuline and N-methylcoclaurine in the Annonaceae family using liquid chromatography-tandem mass spectrometry. J Chromatogr B 2004;806:75–8.
- González-Esquinca AR, De-La-Cruz-Chacón I, Castro-Moreno M, Orozco-Castillo JA, Riley-Saldaña CA. Alkaloids and acetogenins in Annonaceae development: biological considerations. Rev Bras Frutic 2014;36:01–16.
- Hess MG, Barralis H, Bleiholder L, Buhr T, Eggers H, Hack RS. Use of the extended BBCH-scale – general for the description of the growth stages of mono- and dicotyledonous weed species. Weed Res 1997;37:441–3.
- Reglas Internacionales para el ensayo de semillas 1976. Ministerio de Agricultura. Dirección General de Producción Agraria, Madrid 1976;23–32.
- González-Esquinca AR. Contribución al estudio del Genero *Annona* (Annonaceae), análisis fitoquímico de tres especies del estado de Chiapas. Tesis. UNAM 2001.
- Pérez Gutiérrez RM. Handbook of compounds with cytotoxic activity isolated from plants. New York: Nova Science Publishers, Inc., 2007.
- Rieser MJ, Gu ZM, Fang XP, Zeng L, Wood KV, McLaughlin JL. Five novel mono-tetrahydrofuran ring acetogenins from the seeds of *Annona muricata*. J Nat Prod 1996;59:100–8.
- Alali FA, Xiao-Xi L, McLaughlin JL. Annonaceous acetogenins: recent progress. J Nat Prod 1999;62:504–40.
- Gromek D, Hocquemiller R, Cavé A. Qualitative and quantitative evaluation of annonaceous acetogenins by high performance liquid chromatography. Phytochem Anal 1999;5:133–40.

33. Egydio AP, Valvassoura TA, Santos DY. Geographical variation of isoquinoline alkaloids of *Annona crassiflora* Mart. from Cerrado, Brazil. *Biochem Syst Ecol* 2013;46:145–51.
34. Cavé A, Figadère B, Laurens A, Cortes D. Acetogenins from Annonaceae. *Forsch Chem Org Naturst* 1997;70:81–288.
35. Brechú-Franco AE, Laguna Hernández G, De la Cruz Chacón I, González-Esquínca AR. In situ histochemical localisation of alkaloids and acetogenins in the endosperm and embryonic axis of *Annona macrophyllata* Donn. Sm. Seeds during germination. *Eur J Histochem* 2016;61:2568.
36. Wu FE, Zhao GX, Zeng L, Zhang Y, Schwedler JT, McLaughlin JL, et al. Additional bioactive acetogenins, annomutacin and (2, 4-trans and cis)-10R-annonacin-A-ones, from the leaves of *Annona muricata*. *J Nat Prod* 1995;58:1430–7.
37. Chang FR, Wu YC. Novel cytotoxic annonaceous acetogenins from *Annona muricata*. *J Nat Prod* 2001;64:925–31.
38. Kim GS, Zeng L, Alali F, Rogers LL, Wu FE, Sastrodihardjo S, et al. Muricoreacin and murihexocin C, mono-tetrahydrofuran acetogenins, from the leaves of *Annona muricata*. *Phytochemistry* 1998;49:565–71.
39. Zafra-Polo MC, Figadère B, Gallardo T, Tormo J, Cortes D. Natural acetogenins from Annonaceae, synthesis and mechanisms of action. *Phytochemistry* 1998;48:1087–117.
40. Kim GS, Zeng L, Alali F, Rogers LL, Wu FE, McLaughlin JL, et al. Two new mono-tetrahydrofuran ring acetogenins, annomuricin e and muricapentocin, from the leaves of *Annona muricata*. *J Nat Prod* 1998;61:432–6.
41. Gleye C, Laurens A, Hocquemiller R, Cavé A. Cis-monotetrahydrofuran acetogenins from the roots of *Annona muricata*. *J Nat Prod* 1998;61:576–9.
42. Gleye C, Raynaud S, Fourneau C, Laurens A, Laprévotte O, Serani L, et al. Cohibins C and D, two important metabolites in the biogenesis of acetogenins from *Annona muricata* and *Annona nutans*. *J Nat Prod* 2000;63:1192–6.
43. Liaw X, Chang F, Lin C, Chou C, Chiu H, Wu M, et al. New cytotoxic monotetrahydrofuran annonaceous acetogenins from *Annona muricata*. *J Nat Prod* 2002;65:470–5.
44. Rieser MJ, Kozłowski JF, Wood KV, McLaughlin JL. Muricatacin: a simple biologically active acetogenin derivative from the seeds of *Annona muricata* (Annonaceae). *Tetrahedron Lett* 1991;32:1137–40.
45. Myint SH, Cortes D, Laurens A, Hocquemiller R, Lebeuf M, Cavé A, et al. Solamin, a cytotoxic mono-tetrahydrofuranic γ -lactone acetogenin from *Annona muricata* seeds. *Phytochemistry* 1991;30:3335–8.
46. Chang F, Liaw C, Lin C, Chou C, Chiu H, Wu Y. New adjacent bis-tetrahydrofuran annonaceous acetogenins from *Annona muricata*. *Planta Med* 2003;69:241–6.
47. Luna JD, De Carvalho JM, De Lima MR, Bieber LW, Bento ED, Franck X, et al. Acetogenins in *Annona muricata* L. (Annonaceae) leaves are potent molluscicides. *Nat Prod Res* 2006;20:253–7.
48. Zeng L, Wu FE, Oberlies NH, McLaughlin JL, Sastrodihardjo S. Five new monotetrahydrofuran ring acetogenins from the leaves of *Annona muricata*. *J Nat Prod* 1996;59:1035–42.
49. Cortes D, Figadère B, Cavé A. Bis-tetrahydrofuran acetogenins from Annonaceae. *Phytochemistry* 1993;32:1467–73.
50. Patel MS, Patel JK. A review on a miracle fruits of *Annona muricata*. *J Pharmacogn Phytochem* 2016;5:137–48.
51. González-Coloma A, Guadaño A, De Inés CD, Martínez-Díaz R, Cortes D. Selective action of acetogenin mitochondrial complex I inhibitors. *Z Naturforsch C* 2002;57:1028–34.
52. Álvarez O, Barrachina I, Ayala I, González MC, Moya P, Neske A, et al. Toxic effects of annonaceous acetogenins on *Oncopeltus fasciatus*. *J Pest Sci* 2008;81:85–9.
53. Blessing LD, Colom OÁ, Popich S, Neske A, Bardón A. Antifeedant and toxic effects of acetogenins from *Annona montana* on *Spodoptera frugiperda*. *J Pest Sci* 2010;83:307–10.
54. Dang QL, Kim WK, Nguyen CM, Choi YH, Choi GJ, Jang KS, et al. Nematicidal and antifungal activities of annonaceous acetogenins from *Annona squamosa* against various plant pathogens. *J Agric Food Chem* 2011;59:11160–7.
55. Facchini PJ, De Luca V. Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. *Plant J* 2008;54:763–84.
56. Paulo MD, Barbosa-Filho J, Lima EO, Maia RF, de Cassia R, Barbosa BB, et al. Antimicrobial activity of benzyloisoquinoline alkaloids from *Annona salzmanii* DC. *J Ethnopharmacol* 1992;36:39–41.
57. Hasrat J, Pieters L, de Backer JP, Vauquelin G, Vlietinck A. Screening of medicinal plants from suriname for 5-HT_{1A} ligands: bioactive isoquinoline alkaloids from the fruit of *Annona muricata*. *Phytomedicine* 1997;4:133–40.
58. Leboeuf M, Cavé A, Bhaumik P, Mukherjee B, Mukherjee R. The phytochemistry of the annonaceae. *Phytochemistry* 1980;21:2783–813.
59. Batschauer A, Ehmann B, Schäfer E. Cloning and characterization of a chalcone synthase gene from mustard and its light-dependent expression. *Plant Mol Biol* 1991;16:175–85.
60. Kubasek WL, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM. Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. *Plant Cell* 1992;4:1229–36.
61. Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, et al. Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J* 1995;8:659–71.
62. Kubasek WL, Ausubel FM, Shirley BW. A light-independent developmental mechanism potentiates flavonoid gene expression in *Arabidopsis* seedlings. *Plant J Plant Mol Biol* 1998;37:217–23.
63. Balsevich J, De Luca V, Kurz WG. Altered alkaloid pattern in dark grown seedlings of *Catharanthus roseus*. The isolation and characterization of 4-desacetoxyvindoline: a novel indole alkaloid and proposed precursor of vindoline. *Heterocycles* 1986;24:2415–21.
64. DeLuca V, Balsevich J, Tyler RT, Eilert U, Panchuk BD, Kurz WG. Biosynthesis of indole alkaloids: developmental regulation of the biosynthetic pathway from tabersonine to vindoline in *Catharanthus roseus*. *J Plant Physiol* 1986;125:147–56.
65. Aerts RJ, Gisi D, Carolis E, Luca V, Baumann TW. Methyl jasmonate vapor increases the developmentally controlled synthesis of alkaloids in *Catharanthus* and *Cinchona* seedlings. *Plant J* 1994;5:635–43.
66. Beaudoin GA, Facchini PJ. Benzyloisoquinoline alkaloid biosynthesis in opium poppy. *Planta* 2014;240:19–32.
67. Ljung K. Auxin metabolism and homeostasis during plant development. *Development* 2013;140:943–50.
68. Normanly J. Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. *Cold Spring Harb Perspect Biol* 2010;2:a001594.