ALKALOIDS AND ACETOGENINS IN ANNONACEAE DEVELOPMENT: BIOLOGICAL CONSIDERATIONS

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ABSTRACT - Chemical studies of the plant family Annonaceae have intensified in the last several decades due to the discovery of annonaceous molecules with medicinal potential (e.g., benzylisoquinoline alkaloids and acetogenins). Approximately 500 alkaloids have been identified in 138 Annonaceae species in 43 genera. In addition, until 2004, 593 annonaceous acetogenins (ACGs) had been identified, from 51 species in 13 genera. This suggests that plants from this family allocate important resources to the biosynthesis of these compounds. Despite the diversity of these molecules, their biological roles, including their physiological and/or ecological functions, are not well understood. In this study, it was provided new data describing the variety and distribution of certain alkaloids and ACGs in annonaceous plants in distinct stages of development. The potential relationships among some of these compounds and the seasonally climatic changes occurring in the plant habitat are also discussed. These data will improve our understanding of the secondary metabolism of these pharmacologically important molecules and their expression patterns during development, which will help to determine the optimal growth conditions and harvest times for their production.

Index terms: early development, secondary metabolism, ecological functions, phenology.

ALCALOIDES E ACETOGENINAS NO DESENVOLVIMENTO DE ANNONACEAE: CONSIDERAÇÕES BIOLÓGICAS

RESUMO - Estudos químicos em plantas da família Annonaceae se intensificaram nas últimas décadas, devido à descoberta de moléculas de anonáceas com potencial medicinal (por exemplo, alcalóides benzilisoquinolina e acetogeninas). Aproximadamente 500 alcaloides têm sido identificados em 138 espécies de Annonaceae em 43 géneros. Além disso, até 2004, 593 acetogeninas de anonáceas (ACGs) foram identificados, a partir de 51 espécies em 13 géneros. Isto sugere que as plantas desta família atribuem recursos importantes para a biossíntese destes compostos. Apesar da diversidade destas moléculas, as suas funções biológicas, incluindo as suas funções fisiológicas e/ou ecológicas, não são bem compreendidas. Neste estudo, foram fornecidos novos dados que descrevem a variedade e a distribuição de certos alcaloides e ACGs em anonáceas em fases distintas de desenvolvimento. As possíveis relações entre alguns destes compostos e as alterações climáticas sazonais ocorrendo no habitat são também discutidas. Estes dados vão melhorar a nossa compreensão do metabolismo secundário dessas moléculas farmacologicamente importantes e seus padrões de expressão durante o desenvolvimento, o que ajudará a determinar as condições ideais de crescimento e épocas de colheita para a sua produção.

Termos para indexação: desenvolvimento inicial, metabolismo secundário, funções ecológicas, fenologia.

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The origin of the questions

The Annonaceae is a family of plants widely distributed throughout tropical and subtropical regions and includes approximately 108 genera and 2400 species (CHATROU et al., 2012). This family is assigned to the clade Magnoliid according to the APG III system (2009) and according to Cronquist (1981) it was in subclass Magnoliidae, and order Magnoliales. It was among the first flowering plant families included in the ranalean complex, which is composed of families with older phenotypes. From a chemical perspective, the Annonaceae family has been considered a production centre for benzylisoquinoline alkaloids (CRONQUIST, 1981; WATERMAN, 1984).

The benzylisoquinoline alkaloids, specifically aporphinic alkaloids, are mainly produced by primitive plants of the Ranalean Complex (Table 1). In 2001, a sensu lato (registry of all aporphinic alkaloids) reported 801 aporphinic alkaloids (GONZÁLEZ-ESQUINCA, 2001). Members of the Annonaceae family produced 232 (29%) of these alkaloids, while families in other orders produced 328 (41%). Other families with abundant alkaloid production include the Lauraceae, with 116 reported alkaloids (14.5%), and the Menispermaceae, with 108 (13.5%). The great structural diversity of annonaceous alkaloids underscores the broad metabolic diversity in the Annonaceae family, as well as the importance of this family as a major producer of alkaloids (GONZÁLEZ-ESQUINCA, 2001). These many of these alkaloids have been reported as toxic, with effects on the mammalian central nervous system, including analgesic and narcotic properties (ALLEN et al., 2004, 2005, 2006; GONZÁLEZ-ESQUINCA, 2001). These alkaloids have made a major contribution to alkaloid diversity. The persistent biosynthesis of these products appears to indicate a role for these compounds in physiological, ecological, and/or evolutionary functions that are important for survival of the plants in this family.

The diversity of alkaloids and ACGs in the Annonaceae family suggests that these secondary metabolites may provide an evolutionary advantage. The persistent biosynthesis of these products appears to indicate a role for these compounds in physiological, ecological, and/or evolutionary functions that are important for survival of the plants in this family.

The ACGs appear to be metabolites specific to the Annonaceae family. Therefore, their biosynthetic pathways are expected to have originated in this plant family. On the other hand, the Annonaceae species have made a major contribution to alkaloid diversity. Because different Annonaceae species have distinct physiological and phenological characteristics, the ecological significance of these compounds is likely to be broad.

The diversity of ACGs and alkaloids found in Annonaceae implies that these plants dedicate photosynthetic resources to this biosynthesis and also implies the conservation of a genetic program.
between species. Therefore, the biosynthesis of these molecules is important for the plants producing them. However, the specific roles of these molecules remain unclear. Diverse strategies must therefore be implemented to dissect the function of these metabolites. A first approach involves the determination of their distributions within the plant. Where in the plant are these compounds synthesised? Is there a specific period in the phenological cycle that is especially crucial for their biosynthesis? Are these biosynthetic pathways constitutively active, or are they induced by specific biotic or abiotic factors? What are their ecological and/or physiological roles? These questions reflect the limitations of our knowledge of the biology of these compounds and suggest lines of investigation for future research.

In search of answers

Among the most abundant species of wild or semi-wild Annona from the tropical caducifolia rainforest in the central depression of the Chiapas State, Mexico, are A. diversifolia, A. lutescens, and A. purpurea, and A. muricata is an abundant crop species. These trees produce edible fruit, and they are typically exposed to well-demarcated periods of rain or drought and are therefore subjected to changing environmental conditions (Figure 2). The variations in their phenological and physiological cycles are among the most important differences between the species. For example, the fruiting of A. diversifolia and A. purpurea peaks at the end of rainy periods (i.e., during drought periods), and their seeds have a hard seed coat and long latency. In contrast, A. lutescens bear fruits at the end of the drought season, and their seeds have a soft coat and do not show latency. A. muricata exhibit yet another pattern, fructifying (although to different extents) throughout the whole year, and its seeds do not show latency. In this study, it was used the above mentioned species as models for the study of the secondary metabolism of alkaloids and ACGs.

Isolated compounds such as liriodenine alkaloids (GONZÁLEZ-ESQUINCA, 2001), atherospermidine and lisicamine from A. diversifolia: oxopurpureine and annomontine from Annona purpurea (GONZÁLEZ-ESQUINCA, 2001; REJÓN-ORANTES et al., 2011) (Figure 3), and the ACGs laherradurine, rolliniastatin-1, rolliniastatin-2, and cherimolin-2 from Annona diversifolia and A. lutescens (Figure 2) (DE LA CRUZ, 2001, ABRAJÁN, 2002; SCHLIE-GUZMAN et al., 2009, LUNA-CAZARES; GONZÁLEZ-ESQUINCA, 2010) are found in different organs of the plants, and their distinct localizations have shed light on the functions that these types of molecules may play in the plants that produce them.

Organ-specific variation associated with species ontogeny

The biosynthesis and accumulation of secondary metabolites is commonly associated with plant development (GREGIANINI et al., 2004; BOEGE, 2005; PARRA-GARCÉS et al., 2010; CAROPRESE et al., 2011). Both alkaloids and ACGs were constitutively expressed in the Annona species that were studied, but they were found in different organs and distinct developmental stages. It has been established, for example, that certain alkaloids are produced during the early developmental stages of A. diversifolia, A. lutescens, A. muricata, and A. purpurea. Among our most significant observations, it was found that alkaloids are not synthesised during seed organogenesis, their biosynthesis begins very early during germination (Figure 4), and the chemical profile of each organ is enriched as the seedling completes its development. Variation in the concentration and number of alkaloids in each organ of the seedling is common. Some alkaloids depend only on the reserve mobilisation from endosperm, while others are only synthesised when the seedling becomes photosynthetic. The alkaloid profile from seedlings with six leaves (approximately 60 days after germination) is similar to the alkaloid profile in the adult plant, at least in the case of A. diversifolia (Figure 5) (DE LA CRUZ; GONZÁLEZ-ESQUINCA, 2012, DE LA CRUZ, 2012; ALFARO 2005, CONTRERAS, 2010). In A. diversifolia and A. lutescens, the emergence of the primary root is accompanied by the initiation of liriodenine alkaloid biosynthesis. This compound is detected first in the endosperm, where it persists until the seedling stage, and then in other organs as they begin the differentiation process. The differentiation of the hypocotyl and epicotyl in A. diversifolia, the species that has been best characterised during early development, is accompanied by the production of two additional alkaloids, lisicamine and atherospermidine. The biosynthesis of these three alkaloids in specific stages of development appears to be linked to the degradation of stored proteins (Figure 6) (DE LA CRUZ; GONZÁLEZ-ESQUINCA, 2012, DE LA CRUZ, 2012). The allocation of stored resources for the biosynthesis of these alkaloids suggests that their functions are important for specific stages of development.
The seedlings and adults from the four *Annona* species mentioned above have similar organ-specific alkaloid profiles (e.g., the alkaloids are more prevalent in the roots). However, the presence of alkaloids has been documented throughout the whole phenological cycle. For example, in *A. lutescens*, once the biosynthesis of liriodenine is initiated, this compound can be detected in seedlings and juveniles, as well as throughout the complete annual cycle of the adult plant (Figure 7). However, there are relevant differences in the quantity of this molecule between distinct stages and organs (CASTRO-MORENO et al., 2013). Similar to liriodenine, other alkaloids are present throughout the complete phenological cycle of at least four *Annona* species. This implies a continuous allocation of resources and energy to their biosynthesis, transport, and accumulation.

**Ecological and physiological variation**

The characteristic and dramatic changes between the drought and rainy seasons in the Tropical Deciduous Forest suggest additional ecological implications for the production of alkaloids by plants such as *A. diversifolia* and *A. lutescens*. These environmental changes induce phenological and physiological changes that are also regulated by the genetic program of the plant. It was recorded an exponential increase in the liriodenine content of the roots during the most severe stage of the drought season (Figure 8) (CASTRO-MORENO et al., 2013). During this time, the plants lose their leaves for several different reasons, including the recycling of nutrients during senescence and foliar death processes. The liriodenine in the roots appears to play a role in the development of resistance to drought conditions.

Another potential function of liriodenine is nitrogen storage; the seedlings respond to the availability of nitrogen by accumulating nutrients, mainly in the roots. The biomass production ratio of alkaloids indicates that the production of alkaloids is inversely proportional to the biomass and that nitrogen availability seems to compensate for this effect (Figure 9) (OROZCO, 2009). These data suggest that liriodenine could have more than one function. *Lutescens* Safford during drought and rainy season.

Another important secondary metabolite production pathway is the biosynthesis of ACGs (CAVE et al., 1997; ALALI, 1999; BERMÉJO, 2005), which differs from alkaloid biosynthesis and seems to take place in the plant embryo during embryogenesis. For this reason, it may be possible to isolate ACGs from the seeds of all members of this plant family. ACGs are found in many parts of the plant, while alkaloids are generally not present in the leaves or seeds. The genetic activation of these pathways (alkaloid vs. ACGs biosynthesis) is organ specific.

For example, when *A. lutescens* fructify, the majority of alkaloids (*liriodenine*) are accumulated in the roots, while the ACGs *laherradurine*, *rolliniastatin*-2, and iso-anno-reticuline are accumulated in the seeds and roots (ABRAJAN, 2002).

**Significance**

The presence of liriodenine during early plant development and the serendipitous finding that the seeds of *Annona* species can be contaminated with *Rhizopus stolonifer* and *Aspergillus glaucus* fungi only during the first days of imbibition (these contaminants disappear when the radicle is visible) allowed the evaluation of the antifungal activity of liriodenine in *A. diversifolia*. *In vitro* studies demonstrated that liriodenine completely inhibited the proliferation of both fungi with minimal inhibitory concentrations of 200 μM and 100 μM, respectively (DE LA CRUZ et al., 2011). *In vivo*, this alkaloid can also inhibit at least 23 other phytopathogenic fungi, including *Puccinia graminis*, *Phytophthora infestans*, and *Helminthosporium teres* (HUFFORD et al., 1980). The amounts of liriodenine detected per milligram of tissue are 10 to 100,000-fold larger that the minimal inhibitory concentrations needed, allowing the plant to counteract multiple fungal invasions (Table 2). The early development of *A. diversifolia* is accompanied by the exponential accumulation of liriodenine in all tissues. Concomitantly, a gradual reduction in phytopathogens is observed. This suggests that the seed allocates a portion of its stored resources for the production of antimicrobial substances and implicates liriodenine in an early defense mechanism against phytopathogens as a survival strategy in this species (DE LA CRUZ et al., 2011).

The distribution of ACGs during the early stages of development also supports an important role for secondary metabolites in the Annonaceae. The ACGs *laherradurine* and *rollinitstatin*-2 are distributed throughout all organs of *A. muricata* seedlings. Rollinitstatin-2 is more abundant than *laherradurine*, except in the leaves (Figure 10). Significant amounts of these ACGs are expressed in the roots and leaves during several developmental stages. The plant seems to produce more rollinitstatin-2 in the roots than in the leaves (with a 2-fold greater expression in the roots in 2-leaf seedlings and 26-fold greater.
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expression in 6-leaf seedlings). In contrast, more laherradurine is produced in the leaves (40-fold greater expression in the leaves of 2-leaf seedlings). These differences in ACG concentrations could have multiple explanations: 1) one of the ACGs is more potent, e.g., Coloma et al., 2002 reported that laherradurine had greater activity against Spodoptera littoralis (phytophagous plague) larvae than did rollinstatin-2 (86% and 29.7% mortality, respectively); 2) the plant allocates greater resources to the protection of the leaves because of their photosynthetic function and to protect the roots because of their important nutrient and water transport functions (i.e., roots and leaves should be considered equally valuable to the plant) (KAPLAN et al., 2008); and 3) these ACGs could have specific activities against distinct groups of pathogens. For example, 100 µg/disk of rollinstatin-2 but not laherradurine inhibits Candida albicans (although this fungus is not a phytopathogen), producing an inhibition halo of 15 mm in diameter (RILEY, 2007). These explanations are consistent with the optimal defence theory (MICKEY, 1974), which predicts that because defence mechanisms are expensive for the plant, they should be allocated to the most valuable organs, in this case, the leaves and roots.

The changes observed in these two ACGs during ontogeny, germination, and seedling development may represent a defence strategy that is dependent on the developmental stage and independent of biotic or abiotic factors, with plasticity to permit modulation in the presence of adverse factors. The early developmental model and secondary metabolism of alkaloids and ACGs in Annonaceae provide an opportunity to evaluate distinct plant defence theories (BERENBAUM 1995; ESPINOSA-GARCÍA, 2001; STAMP, 2003). The current theories do not incorporate the impact of early plant development.

Current evidence from Annonaceae support the optimal defence theory (OD) (STAMP, 2003; LANGEHEIM, 2001) and the growth-differentiation balance (GDB) hypothesis. The latter underscores the physiological trade-offs between growth, differentiation, and secondary metabolite production (HERMS; MATTSON, 1992).

Exploration of future opportunities based on current findings: questions

What are the relationships among alkaloid and ACGs biosynthesis, the stage of plant development or specialisation, and environmental factors? Why are these compounds produced mostly in Annonaceae?

The exploration of alkaloid dynamics and ACG accumulation in the different tissues of plants that live in changing environments will provide important contributions to our understanding of the physiological and ecological responses of these species.
### TABLE I - Alkaloids in Ranalean Complex.

<table>
<thead>
<tr>
<th>ORDEN</th>
<th>MAG</th>
<th>LAU</th>
<th>NYM</th>
<th>RAN</th>
<th>PAP</th>
<th>AR1</th>
<th>SAU</th>
<th>PIP</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAN</td>
<td>EUP</td>
<td>MAG</td>
<td>ANN</td>
<td>LAU</td>
<td>HER</td>
<td>MON</td>
<td>NEL</td>
<td>RAN</td>
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<tr>
<td>Proaporphins</td>
<td>5</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Aporphins</td>
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<td></td>
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<tr>
<td>4,7-oxo substituted Aporphins</td>
<td>1</td>
<td>14</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7, 7- Dimethyl Aporphyns</td>
<td>3</td>
<td></td>
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</tr>
<tr>
<td>7-Hydroxy-7-Methyl porphins</td>
<td>3</td>
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<td></td>
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<td></td>
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<tr>
<td>Dehydro Aporphins</td>
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<tr>
<td>4, 5-Dioxo Aporphins</td>
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<td></td>
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<tr>
<td>Oxo Aporphins</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Tetrahydro Protoberine</td>
<td></td>
<td>11</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Phenanthrene</td>
<td></td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total Alkaloids</td>
<td></td>
<td>54</td>
<td>232</td>
<td>116</td>
<td>40</td>
<td>40</td>
<td>10</td>
<td>54</td>
<td>108</td>
</tr>
</tbody>
</table>

CAN: Canellaceae; EUP: Eupomatiaceae; MAG: Magnoliaceae; ANN: Annonaceae; LAU: Lauraceae; HER: Hernandiaceae; MON: Monimiaceae; NEL: Nelumbonaceae; RAN: Ranunculaceae; MEN: Menispermaceae; BER: Berberidaceae; FUM: Fumariaceae; PAP: Papaveraceae; ARI: Aristolochiaceae; SAU: Saururaceae; PIP: Piperaceae.
FIGURE 1 - Phylogenetic distribution of alkaloid liriodenine.
### TABLE 2- Comparison between liriodenine content in tissues and antifungal activity.

<table>
<thead>
<tr>
<th>Specie/Tissue</th>
<th>µmol /g dried plant</th>
<th>Fungi</th>
<th>CI µmol/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. lutescens</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root 2 year-young plant</td>
<td>775</td>
<td><em>Aspergillus fumigatus</em>&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.09</td>
</tr>
<tr>
<td>Root mature plant</td>
<td>378</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. diversifolia</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>root seedling with two leaves</td>
<td>1.50</td>
<td><em>Laetiporus sulphureus</em>&lt;sup&gt;10&lt;/sup&gt;</td>
<td>0.007</td>
</tr>
<tr>
<td>root seedling with six leaves</td>
<td>2.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mitrephora glabra</strong>&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>stem bark mature plant</td>
<td>0.04</td>
<td><em>Lenzites betulina</em>&lt;sup&gt;10&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Rollinia mucosa</strong>&lt;sup&gt;4&lt;/sup&gt;</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>leaves mature plant</td>
<td>0.04</td>
<td><em>Trametes versicolor</em>&lt;sup&gt;11&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Fissistigma latifolium</strong>&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>stem bark mature plant</td>
<td>0.06</td>
<td><em>Aspergillus glaucus</em>&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Rollinia leptopetala</strong>&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>root bark mature plant</td>
<td>0.46</td>
<td><em>Rhizopus stolonifer</em>&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.2</td>
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<tr>
<td><strong>A. reticulata</strong>&lt;sup&gt;7&lt;/sup&gt;</td>
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<tr>
<td>root bark mature plant</td>
<td>0.18</td>
<td><em>Botrytis faba</em>&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Mollinedia glaziovii</strong>&lt;sup&gt;8&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>stem bark in fruiting plant</td>
<td>735</td>
<td><em>Syncephalastrum racemosum</em>&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>M. gigliana</strong>&lt;sup&gt;8&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td>stem bark in vegetative plant</td>
<td>249</td>
<td><em>Pyricularia oryzae</em>&lt;sup&gt;11&lt;/sup&gt;</td>
<td>0.14</td>
</tr>
</tbody>
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References: 1Castro-Moreno et al., 2013; 2De la Cruz y González-Esquinca, 2012; 3Li et al., 2009; 4De Lima et al., 2012; 5Alias et al., 2010; 6Feitosa, et al., 2009; 7Suresh et al., 2012. 8Moreira y Leitao, 2001, 9Hufford et al., 1980; 10Wu et al., 2011; 11Khan et al., 2002; 12De la Cruz et al., 2011.

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**FIGURE 2-** Phenology of *Annona* species.
FIGURE 3- Alkaloids and acetogenins isolated from *Annona* species.

FIGURE 4- Alkaloids biosynthesis during early development in *Annona* species.
FIGURE 5- Alkaloids accumulation in seedlings and adults plants of *Annona diversifolia*, *Annona muricata* and *Annona lutescens*. Seedlings (A, B and C) and adults plants (D, E y F).
FIGURE 6 - Metabolic relation between reserve mobilization and the liriodenine alkaloid biosynthesis in early development Annona diversifolia Safford.

FIGURE 7 - Ontogenetic accumulation of alkaloids in Annona lutescens Safford.

FIGURE 8 - Liriodenine alkaloid in Annona lutescens Safford during drought and rainy season.
FIGURE 9 - Comparison between in biomass production and alkaloid accumulation in *Annona diversifolia* Safford seedlings. Seedlings with leaves in media cultures with nitrogen variation.

FIGURE 10 - Ontogenetic variation of acetogenins in early development of *Annona muricata* L. A. roots, B. stem bark. C. leaves.
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