Variation of Total Nitrogen, Non-protein Nitrogen Content, and Types of Alkaloids at Different Stages of Development in *Erythrina americana* Seeds†

R. García-Mateos,‡ B. Lucas, M. Zendejas, M. Soto-Hernández,‡ M. Martínez,§ and A. Sotelo*

Departamento de Farmacia, División de Estudios de Posgrado, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, C.P. 04510, México, D.F.

The flowers, string beans, seeds, and pods (without seeds) of *Erythrina americana* were studied in connection with the protein nitrogen, non-protein nitrogen content, and type of alkaloid at different stages of development of the fruit. The protein nitrogen from flowers to mature seeds increased, while the levels of non-protein nitrogen decreased with maturation. The alkaloids accumulated not only at the end of maturation in the seeds but also in young tissues. On a dried basis, a high content of alkaloids was observed in flowers and dry seeds in comparison to low levels in dry pods. The highest concentrations were found in the mature seeds. The GC/MS analysis showed the presence of β-erythroidine, which was the major alkaloid in all the tissues examined. Erysodine and erysovine were found in the mature and dry tissues but not in flowers. *N*-Oxide alkaloids were only found in mature tissues.

Keywords: *Alkaloids; development; erysodine; erysovine; Erythrina americana; gas chromatography−mass spectrometry; Leguminosae; N-oxides; non-protein nitrogen; physiology; total nitrogen*

INTRODUCTION

Erythrina is a large genus of the Leguminosae family, comprising a wide range of morphological variation and ecological diversity. It is prominent among the trees attracting increasing research and development attention and is distributed throughout the tropics. The greatest concentration of *Erythrina* species is found in southern Mexico (27 species) and Central America (Neill, 1993). Many of the approximately 115 *Erythrina* species are used in agroforestry systems as fences, windbreaks, shade, or support for other plants (Neill, 1993; Russo, 1993). As typical legumes they improve the soil and provide animal fodder, human food, medicine, and wood products. Their unique flowers and seeds also make them popular as ornamental handcrafts (Musálem, 1993). These attributes are diminished by the toxicity of the plants because they accumulate alkaloids in all parts, particularly in the seeds. The *Erythrina* alkaloids can be considered typical for this genus; they have unusual structural features and exhibit a restricted distribution within the Leguminosae. The flowers and seeds are rich in alkaloids as in *Erythrina americana* (Aguilar *et al.*, 1981; Cromwell, 1955), and this suggests that these are possible sites of accumulation.

On the other hand, several physiological changes are involved in the development and the maturation process of fruit and seeds. These changes are associated with hormonal factors as well as with accumulation of diverse other chemical compounds (Hobson, 1993; Salisbury and Ross, 1994; Taiz and Zeiger, 1991). During the development of the fruit, there is translocation of nitrogenous products such as amino acids, alkaloids, and amides which account for these compounds being the major nitrogenous compounds in the mature seeds and in the fruit. The concentration of free amino acids decreases when the stored protein and other nitrogenous compounds such as alkaloids are formed (Salisbury and Ross, 1994).

Leguminosae seeds are rich in protein, and in some species the presence of alkaloids has been shown. The presence of alkaloids in flowers and seeds of *Erythrina* species has also been described. Romeo and Bell (1974) point out that in this species the amino acid content is usually in inverse proportion to alkaloid yield: the higher the amino acid content the lower the amount of alkaloids. However, Payne and Foley (1992) point out that this depends on the genetic variability of some clones. They found a correlation between the total alkaloid content and total nitrogen or protein content. Sotelo *et al.* (1993) showed that seeds of *E. americana* contain a high protein content and a characteristic alkaloid profile of the species.

Several researchers stated that in some species the alkaloids are found in the seeds, approximately from 0.05 to 0.1%. Although, the alkaloids have been localized in roots, bark, leaves, and flowers (Dyke and Quessy, 1981; Sotelo *et al.*, 1993), Robinson (1979) pointed out that the development and maturation process in a plant can affect the synthesis of alkaloids, according to their relative concentration and the diversity of structures. Waller and Nowacki (1978) assumed that during the morphological and physiological processes in the plant, the alkaloidal profile can be modified.

On the other hand, it has been found that in legumes, the alkaloids and their concentrations can be changed

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* Author to whom correspondence should be addressed (telephone, 5-622-53-33; fax, 622-53-29; e-mail, angela@servidor.unam.mx).

‡ Present address: Colegio de Postgraduados, Montecillo, C.P. 56230, Mexico.

§ Present address: Instituto de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, C.P. 04510, México, D.F.

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during maturation. There has been a deal of controversy as to whether the expression of secondary plant products such as alkaloids is under genetic or environmental control. In the revised literature, it is commonly observed that the alkaloidal composition of a plant may vary with environmental conditions such as soil nutrient level, soil type, pH, altitude, drainage, sunlight and stress. However, no correlations have been found between alkaloid content and the environmental changes mentioned above (Payne and Foley, 1992).

Very few studies have been done on the changes of alkaloid content and type during the development of the fruit, and nothing has been reported in the revised literature on these changes in *Erythrina* species. The objective of the present work was to determine the changes in nitrogen concentration and the variation of the alkaloid types during the development of the fruit from the flower to the dry seed.

MATERIALS AND METHODS

Materials. Flowers, string beans, seeds, and pods in different stages of development were collected at the following intervals: flower → string beans (whole pod formed), 0−30 days; string beans → mature pods (without seeds) and seeds, 31−45 days; mature pods and seeds → dry pods and seeds, 46−96 days; the process of maturation lasted approximately 96 days. The samples were collected from plants grown in the wild from the gardens of the Faculty of Chemistry at the University Campus, Mexico City, and their authenticity was certified by a botanist from the Herbarium of the Colegio de Postgraduados, Montecillo, Estado de Mexico.

All the tissues were air-dried, milled separately, and extracted with hexane by Soxhlet extraction for 48 h.

Determination of Total Nitrogen. This determination was done according to the technique described by AOAC (1990).

Determination of Non-protein Nitrogen. The analysis was carried out also by the Kjeldahl method after the protein separation using tungstic reagent, according to the method described by Caraway (1958) and Light and Smith (1963). Non-protein nitrogen was obtained by calculating the difference between total and protein nitrogen.

Extraction of the Alkaloids. Alkaloids were extracted from small samples of tissue by the same method as that used previously (Games *et al.*, 1974). Alkaloids in the hexane fraction were washed with 1 M sulfuric acid (3 × 50 mL), and the aqueous acidic phase was adjusted to pH 8 using solid $NaHCO_3$. Finally, extraction with CH_2Cl_2 (3 × 100 mL) gave the hexane fraction "free" alkaloid.

The defatted flour of each fraction was next extracted in a Soxhlet for 48 h with MeOH, the extract evaporated under vacuum, and the residue taken up in sulfuric acid. The acidic solution was extracted with CH_2Cl_2 to remove traces of fat. The aqueous phase was basified with $NaHCO_3$ to pH 8 and was extracted with CH_2Cl_2 (3 × 100 mL) to give a methanolic fraction of the "free" alkaloids. The remaining aqueous phase was reacidified to pH 2 with hydrochloric acid and heated at reflux at 60−70 °C for 3 h to hydrolyze the esterified alkaloids. The process of extraction was repeated after basification to pH 8 to yield the "liberated" alkaloid fraction. The solvent of each sample was evaporated, and the residue was dried in a desiccator and weighed.

Gas Chromatography−Mass Spectrometry (GC/MS). The crude alkaloid mixture (2 mg) was derivatized as trimethylsilyl (TMS) derivatives by treatment for 30 min with *N*,*O*-bis(trimethylsilyl)acetamide (25 μL) in acetonitrile (25 μL), using a Teflon-lined screw-cap vial to prevent evaporation (Games *et al.*, 1974). The identification of alkaloids was done by comparison with authentic samples (erysodine, erysovine, erythraline, α- and β-erythroidines, erysopine), interpretation of mass spectral characteristics, and use of reference data.

Mass spectra were determined on a JMS-AX 505 HA (JEOL) mass spectrometer coupled with a GC Hewlett Packard 5890, Series II, instrument equipped with a flame ionization detector and a PAS 1701 silicone capillary column (25 m × 0.32 mm × 0.25 μm; Hewlett Packard, Palo Alto, CA) via a two-stage Watson-Biemann separator. The temperature of the ion source was 220 °C, and the accelerating and ionizing potentials were 3 kV and 70 eV, respectively.

Table 1. Content of Nitrogen and Alkaloids during Development of the Fruit of *E. americana*

component	stage of development: flowers	string beans	mature seeds	mature pods[a]	dry seeds	dry pods
humidity[b]	90.30	87.83	78.90	87.60	5.67	7.56
total nitrogen[c]	3.42	3.17	4.92	1.80	5.47	1.64
protein nitrogen[c]	1.75	2.24	4.38	1.06	5.17	1.23
non-protein nitrogen[c]	1.67	0.93	0.54	0.74	0.30	0.41
total alkaloids[d]	1.10	0.39	0.17	0.64	1.05	0.27
free alkaloids[d,e]	1.02	0.21	0.15	0.58	0.75	0.13
liberated alkaloids[d]	0.08	0.18	0.02	0.06	0.30	0.14
alkaloids/non-protein nitrogen[f]	0.66	0.42	0.32	0.86	3.50	0.66

[a] Mature pods (without seeds). [b] g of water/100 g of raw material. [c] g of nitrogen/100 g of dry material. [d] g of alkaloids/100 g of dry material. [e] Free alkaloids (hexane fraction + methanol fraction). [f] Total alkaloids and non-protein nitrogen ratio.

RESULTS AND DISCUSSION

Table 1 shows the results of percent humidity, total nitrogen, protein nitrogen, and non-protein nitrogen content at different stages of development of the fruit. The highest moisture content was found in the flower which remarkably decreased in the dry seed. It was found that protein nitrogen was increased during seed development, from the flower stage to the dry seed, while the non-protein nitrogen decreased during the maturation of the fruit.

The alkaloid yield of each tissue examined is also shown in Table 1. The "free" alkaloids (hexane soluble and methanol soluble fractions) were the most abundant in all the tissues examined, except in the dry pods, where the "liberated" alkaloids were present in the same proportion as the "free" alkaloids. We also included in the studies the analysis of the "hexane" fraction, since it is known that the hexane fraction contains significant quantities of alkaloids (Hargreaves *et al.*, 1974; Sotelo *et al.*, 1993).

The quantitative analysis of total alkaloids ("free" and "liberated" alkaloids) showed interesting trends in the variation of alkaloid concentrations during the process of the fruit maturation. The flowers, for example, synthesized moderate levels of alkaloids mainly as "free" alkaloids, declined at the string bean stage, and again attained their highest accumulation in dry seeds. This means that the alkaloids increase during seed maturation with corresponding decrement of non-protein nitrogen content. It suggests the transformation of free amino acid into alkaloids (Romeo and Bell, 1974).

The absolute values shown in Table 1 did not give any information about the "translocation" of the alkaloids during the development of the seeds. However, when the total alkaloid content was related to non-protein nitrogen, a clear tendency was found of increasing relative concentration of the alkaloids from the flower to dry seed, an effect not observed in separated pod material, which is shown in Figure 1. This agrees with the statement of Waller and Nowacki (1978), who point out that a change of an alkaloid spectrum in a plant organ during its development often indicates at least one phenomenon: *de novo* synthesis, translocation,

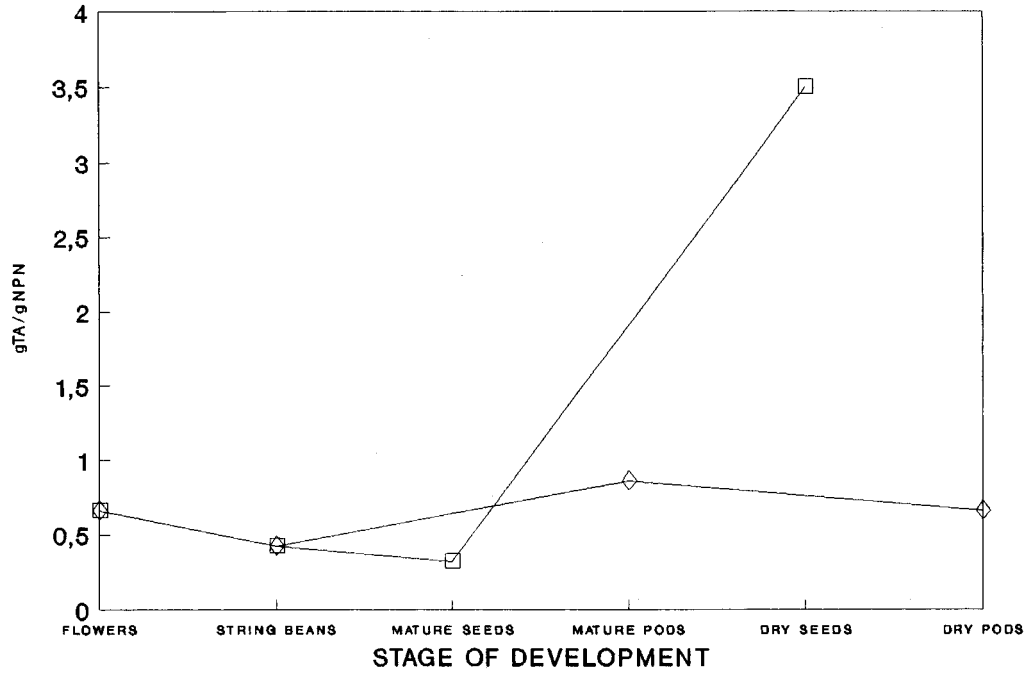

Figure 1. Alkaloidal content and non-protein nitrogen ratio during the development of the fruit of *E. americana* (g of total alkaloids/g of non-protein nitrogen, g of TA/g of NPN): (□) whole fruits or seeds and (◊) pods without seeds.

or degradation. This was observed during seed development but not in the pod material.

It is important to consider the contribution of the moisture in flowers and seeds (Table 1). The lower alkaloidal content in the flowers and the high solubility of these alkaloids in water when the fresh flowers (ca. 0.1%) are boiled make the flowers edible as traditional Mexican food.

The alkaloids of the methanol soluble fractions of *E. americana* were identified by GC/MS. In the analysis it was convenient to divide the alkaloids into two groups: those which contain a conjugated 1,6-diene system (*e.g.*, erysodine, **1**) and those which contain an isolated 1(6)-double bond (*e.g.*, erythratidine, **6**); the former group comprises the aromatic diene alkaloids (erysodine (**1**), erysovine (**2**), erysotrine (**3**), 11-MeO-erythraline (**4**), and 11-OH-erysovine (**5**). The structures of the identified alkaloids are showed in Figure 2. The mass spectra of all these alkaloids showed essentially the same fragmentation pattern. The major peaks were at M^+, $M^+ - 15$, $M^+ - 31$, $M^+ - 58$, $M^+ - 72$, and $M^+ - 85$ (Boar and Widdowson, 1970).

The nonaromatic α- and *β*-erythroidines (**9** and **10**, respectively) belong to the diene group and fragment by loss of the methoxyl substituent at C-3. α-Erythroidine (**9**) showed a M^+ *m*/*z* 273 and a major fragment ion at *m*/*z* 242 ($M^+ - 31$). The derivative of *β*-erythroidine·TMS showed a similar fragmentation pattern except for the presence of an intense ion at *m*/*z* 73 (M^+), 345, and 130, due to the fragment C_2HO-TMS. The presence of the rare alkaloids erythartine *N*-oxide (**7**) and erythristemine *N*-oxide (**8**) also was observed; they showed the typical fragmentation pattern of the diene aromatic alkaloids besides the fragment of $M^+ - 16$ that supported the evidence of the N-O residue.

The fragmentation of the second group of *Erythrina* alkaloids (*e.g.*, erythratidine, **6**), those having a 1(6)-double bond, was more complex and varied than those of the above group. The ions $M^+ - 15$ and $M^+ - 31$ were of relatively minor importance, but the ion $M^+ - 58$ that correspond to a retro-Diels−Alder reaction in ring A was the major peak in this group.

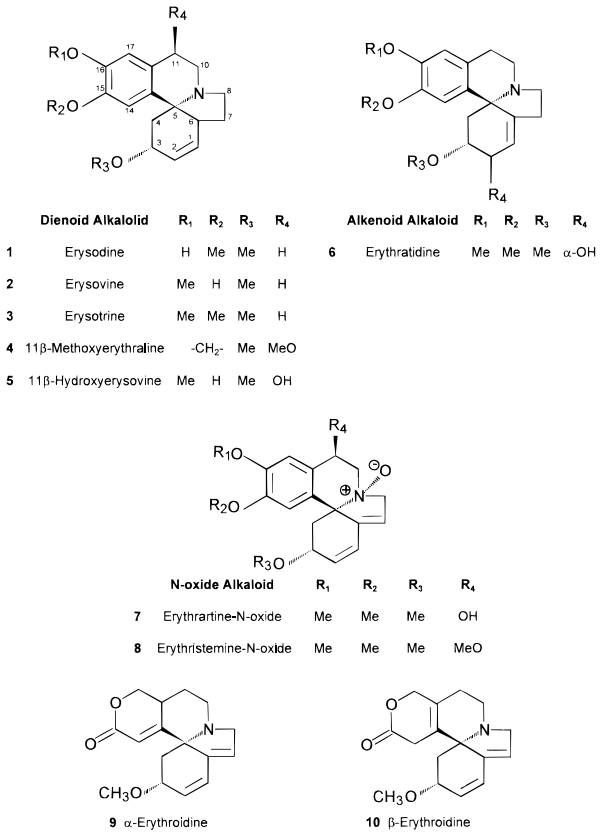

Figure 2. Structures of *E. americana* alkaloids found in the different stages of development of the fruit.

Finally, the presence of an unidentified alkaloid (**11**) was observed. It showed MW 329 and major peaks at 329 (M^+), 319 (100), 113....73. It probably belongs to the second group of alkaloids, and additional work is being done in order to elucidate its structure.

In the GC/MS examination of the hexane fraction was identified the presence of α- and *β*-erythroidines in all tissues examined, and they are included in the "free" alkaloids fraction. The "liberated" fraction afforded only

Table 2. Content of Alkaloids with Development Stages of *E. americana*

	alkaloids										
tissue	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
flowers								+	+	+	+
string beans							+	+	+	+	+
mature pods	+	+	+				+	+	+	+	
mature seeds	+						+		+		+
dry pods	+			+	+	+			+	+	+
dry seeds	+	+							+	+	

erysodine/erysovine. The same pattern of alkaloids is observed in the *Erythrina* of the New World (Hargreaves *et al.*, 1974).

The distribution of the alkaloids detected in the different stages of development of the fruit is shown in Table 2. The dry pods showed seven different alkaloids, whereas flowers and dry seeds showed only four alkaloids (compounds **4**−**6** were only found in dry pods). Erysodine (**1**), erysovine (**2**), and erysotrine (**3**) were found in mature pods. The majority of methylated alkaloids present in the early stages disappeared during maturation. Compounds hydroxylated in position C-11 are rarely found in this species, *e.g.*, 11β-methoxyerythraline (**4**) and 11β-hydroxyerysovine (**5**) were only present in dry pods.

The flowers showed the presence of α- and β-erythroidines, as described in *E. americana* by Aguilar *et al.* (1981) and in seeds by Sotelo *et al.* (1993) and Hargreaves *et al.* (1974). The presence of erysopine (**1**) is common in this species together with erysovine (**2**) (Abdullah, 1980; Hargreaves *et al.*, 1974; Sotelo *et al.*, 1993), but it has not been described in mature tissues, although the presence of compounds oxygenated at C-11 suggests a metabolism of degradation in the final pass of the maturation to dry pods. The alkenoid alkaloid erythratidine (**6**) has not been described in flowers and seeds of *E. americana.* Hargreaves *et al.* (1974) pointed out that the **6** type alkenoid is a biogenic precursor of the **1**−**5** type dienoid alkaloid; however, in the present study this compound was detected in the dry pod (without seeds).

Unexpected compounds were also observed, for example, the alkaloid *N*-oxides **7** and **8**, in string beans, mature tissues, and flowers. They have not been described in *E. americana* but were reported in flowers and seeds of *Erythrina mulungu* and *Erythrina berteroana* (Sarragiotto *et al.*, 1981; Soto, 1989; Soto and Jackson, 1994). These authors have also pointed out that these *N*-oxides are, in fact, natural products and not artifacts (Phillipson and Handa, 1978). Toppel *et al.* (1988) point out that tertiary alkaloids (*e.g.*, pyrrolizidine alkaloids from *Crotalaria scassellatii*) are rapidly converted into respective *N*-oxides during the first days of seeding development. This evidence shows (Toppel *et al.*, 1988) that the very polar saltlike *N*-oxides are translocated to the place where they are stored and the tertiary alkaloids are accumulated only in dry seeds, and perhaps for this reason, they were not found in mature seeds in this study.

These results confirm that α-erythroidine (**9**) and β-erythroidine (**10**) are found in all stages of fruit maturation including flowers (Aguilar *et al.*, 1981; Sotelo *et al.*, 1993). The explanation of this pattern can be inferred through their biosynthesis because, as Barton *et al.* (1974) pointed out, erysodine is one of the main alkaloids that remains until the end of biosynthesis. Only alkylation or dealkylation of the phenolic group or decarboxylation of ring D can determine the transformation of erysodine as a precursor of the lactonic alkaloid β-erythroidine (**10**) through several intermediate stages (Dyke and Quessy, 1981); so this compound is the final product of the biosynthetic pathway in the examined tissues. It is supposed that of all the possible biosynthetic precursors, mainly the aromatic types of *Erythrina* alkaloids are shunted into this pathway and are converted mainly to β-erythroidine (**10**). Accordingly the aromatic *Erythrina* alkaloid precursors may not be present or detected in significant amounts because they may be rapidly converted to β-erythroidine (**10**) (Payne and Foley, 1992).

CONCLUSIONS

The main aim of this work was to show the alkaloidal, protein, and non-protein content variation in different stages of development of the fruit of *E. americana.* Alkaloid accumulations were not only observed at the end of maturation of seeds but also in young growing tissues like flowers, mature pods, and seeds. The alkaloids and proteins increased simultaneously during fruit maturation and are in inverse relation to the non-protein nitrogen content.

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