Original Article

Botanical and genetic characters of *Erythrina × neillii* cultivated in Egypt

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ABSTRACT

Erythrina × neillii Mabberley & Lorence, Fabaceae, is a sterile hybrid between *E. herbacea* L. and *E. humeana* Spreng. Nothing was traced about its genetic, macro and micromorphology. Therefore, it was deemed of interest to study its botanical characters, in addition to the DNA fingerprint to help in the identification of the plant. The anatomical characters of the old stem and its bark are characterized by the presence of cork cells, bast fibers and sclereids. Pericycle is sclerenchymatous forming crystal sheath. The epidermises of the leaf and young stem are characterized by the presence of anomocytic and paracytic stomata, non-glandular, unicellular and multicellular two armed hairs, and glandular club shaped hair. Calcium oxalate is present in the form of crystal sheath and prisms. Secretory cavities are distributed in the phloem and cortex. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used as one of the molecular methods to differentiate between the samples of *Erythrina*. The DNA of *Erythrina* was extracted and analyzed using seven-mer random primers. Randomly Amplified Polymorphic DNA were recognized. This characterization allows certification of the authenticity of *Erythrina × neillii*, in order to provide quality control for the plant.

Introduction

Erythrina genus, belonging to family Fabaceae, comprises about 200 species, which are distributed in tropical and subtropical regions worldwide. They are trees, growing up to 30 m (98 ft.) in height. The generic name is derived from the Greek word (*erythros*), meaning "red," referring to the flower color of certain species (Gledhill, 2008). *Erythrina* has been used in folk medicine for treatment of insomnia, malaria, venereal diseases, asthma, and toothache (de Araújo-Júnior et al., 2012). Plants belonging to the *Erythrina* genus are sources of tetracyclic alkaloids, flavonoids, especially isoflavones, coumarins and saponins (Soto-Hernández et al., 2012).

Erythrina × neillii Mabberley & Lorence is a hybrid derived from a cross between *E. herbacea* L. and *E. humeana* Spreng. at the National Tropical Botanical Garden. The English name is Neill's coral tree which is named after its synthesizer, David Neill, associate curator at Missouri botanical Garden. This sterile hybrid produces attractive bright red flowers but does not produce fruits or seeds (Neill, 1984).

Regarding the macro, micromorphology and genetic characters of *Erythrina × neilli* nothing was traced, therefore, it was deemed of interest to study its botanical and genetic characters to establish criteria that would be helpful for its discrimination from other *Erythrina* species and ensuring safety for commercial pharmacological uses.

Materials and methods

Plant material

Leaves, stem, and bark of *Erythrina × neillii* Mabberley & Lorence, Fabaceae, were collected during the flowering period in October 2012 from El-Zohria garden, Cairo. It was kindly identified by Dr. Gwilym P. Lewis, Legume Research Leader, Comparative Plant & Fungal Biology. Royal Botanic Gardens, Kew, UK and approved by Prof. Dr. David Neill, director of the Unit of Conservation and Wildlife Management of the Amazon State University, Kew Royal Botanic Gardens. The voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, MSA University under registration number RS020 and duplicates housed in the Kew Herbarium (K).

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Methodology for botanical study

The anatomical investigations were performed on cross sections of the plant organs under study which were preserved in 70% alcohol containing 5% glycerol and on air-dried finely powdered samples. The photographs were taken using optical microscope coupled with a digital camera DfC295. Histochemical tests were performed for the presence of lipophilic substances using Sudan III, phenolic compounds with ferric chloride, alkaloids with Dragendorff reagent and starch with iodine.

Extraction and purification of genomic DNA

A half-gram of young green leaves of $E\times$ *neillii*, *E. indica*, *E. speciosa*, *E. variegata* and *E. japonica* was collected and quickly frozen in liquid nitrogen and then ground using mortar and pestle. A modified CTAB (hexadecyltrimethyl ammonium bromide) procedure based on the protocol of Porebski et al. (1997) was adopted for obtaining a good quality of total DNA.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD-PCR reactions

A set of seven-mer random primers was used in the detection of polymorphism among the five plant accessions, having the following sequences: OPA-11: TCTGTGCTGG, OPA-10: GAAACGGGTG, OPB-06: CATCCCCCTG, OPB-07: TGCTCTGCCC, OPD-14: TTCCCC-CGCT, OPD-15: TTTGCCCGGA, OPC-01: TTCGAGCCAG. RAPD was carried out according to the procedure given by Williams et al. (1990).

Thermocycling profile and detection of the PCR products

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94 °C. Each cycle consisted of a denaturation step at 94 °C for 1 min, an annealing step at 36 °C for 1 min, and an elongation step at 72 °C for 1.5 min. The primer extension segment was extended to 7 min at 72 °C in the final cycle. The amplification products were resolved by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in $1\times$ TBE buffer at 95 volts. PCR products were visualized under UV light and photographed using a Polaroid camera. RAPD-PCR-amplified fragments were scored as present (1) or absent (0). Only clear and major bands were scored (Collard and Mackill, 2009).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Total cellular proteins were extracted by resuspending the plant extract in one volume of sample Tris-EDTA buffer and incubating for 5 min at 100 °C and then for 2 min on ice. Cell debris were removed by centrifugation at maximum speed ($22,640\times g$) for 2 min. The supernatant containing total cellular protein was loaded onto 12% (w/v) SDS-PAGE gels and was run at 45 mA for 1 h, then fixed and stained with Coomassie brilliant blue (Laemmli, 1970).

Electrophoresis of protein gel

The gel apparatus was assembled and the lower and upper chambers were filled with the tank buffer. A drop of bromophenol blue was added as a tracking dye. A Hamilton syringe was used to load an equal volume of cellular protein (10 μl) in each well. Pre-stained molecular weight protein marker from Bio-Rad, was applied to the gel. Electrophoresis was carried out at about 100 V ($\approx$20–30 mA) in $1\times$ Tris/glycine-SDS-running buffer until the tracking dye reached the bottom of the gel ($\approx$1.5–2 h) (Laemmli, 1970).

Staining and destaining of the protein gel

After electrophoresis, the gel was stained with 50 ml of staining solution (0.125% coomassie blue R-250, 50% methanol and 10% acetic acid) for 30–45 min with shaking at room temperature and destained in destaining solution (40% methanol and 7% acetic acid). The gel was placed between two sheets of cellophane membrane and dried on gel drier for 2 h and then photographed (Laemmli, 1970).

Results

Macroscopic features

Erythrina $\times$ *neillii* is an up to 3 m height tree, having a thick erect trunk with monopodial branching and carrying terminal panicle red inflorescence (Fig. 1A). The old stem is woody, dark brown in color with wrinkled bark that breaks with short fibrous fracture. The outer surface of the bark is dark brown in color, showing lenticels, longitudinal wrinkles and cracked at irregular intervals, while the inner surface is smooth and light brown in color (Fig. 1B). The young stems and branches are yellowish green, pubescent, with stout conical spines (Fig. 1C). The petiole is more or less cylindrical, yellowish green in color and pubescent showing a swollen pulvinus at its connection with the stem. The leaves are alternate, compound and trifoliate. The lower pair leaflets are smaller than the apical one (Fig. 1D and E). The leaflets are ovate rhomboidal with an entire margin and a symmetric base. The lamina has an acute apex, reticulate pinnate venation, and a very short petiolule with large, cup-shaped gland. The leaflets have smooth and shiny surfaces and coriaceous texture (Fig. 1F and G).

Microscopic features

The young stem

In cross-sectional view (Fig. 2A), the young stem is nearly circular in outline. It is formed of an outer epidermis followed by narrow cortex. Pericycle is parenchymatous interrupted by groups of fibers. The vascular tissue is formed of a complete ring of collateral vascular bundles followed by a wide pith.

The epidermis (Fig. 2B and C) is polygonal and slightly elongated cells with almost straight, beaded anticlinal walls, and covered with thick smooth cuticle showing anomocytic and paracytic stomata. Two types of hairs: glandular club-shaped with a spherical multicellular head and short unicellular stalk and nonglandular, unicellular to multicellular with equal or unequal two arms.

The cortex (Fig. 2B) is formed of 3–4 layers of collenchymatous cells followed by 6–8 layers of collapsed thin-walled parenchymatous cells. Styloid prisms of calcium oxalate and starch granules are frequently distributed in the cortical parenchyma cells. The starch is mainly simple and sometimes compound of 2–3 granules; it is spherical to oval in shape with pointed hilum. The endodermis is distinctive, formed of parenchyma lined in a crescent shape and containing starch forming starch sheath. The pericycle is parenchymatous, interrupted by groups of lignified fibers surrounded by parenchyma cells containing prismatic crystals of calcium oxalate forming a crystal sheath. Fibers are usually long with thick lignified straight walls, narrow lumen, and blunt to acute apices (Fig. 2C). The phloem
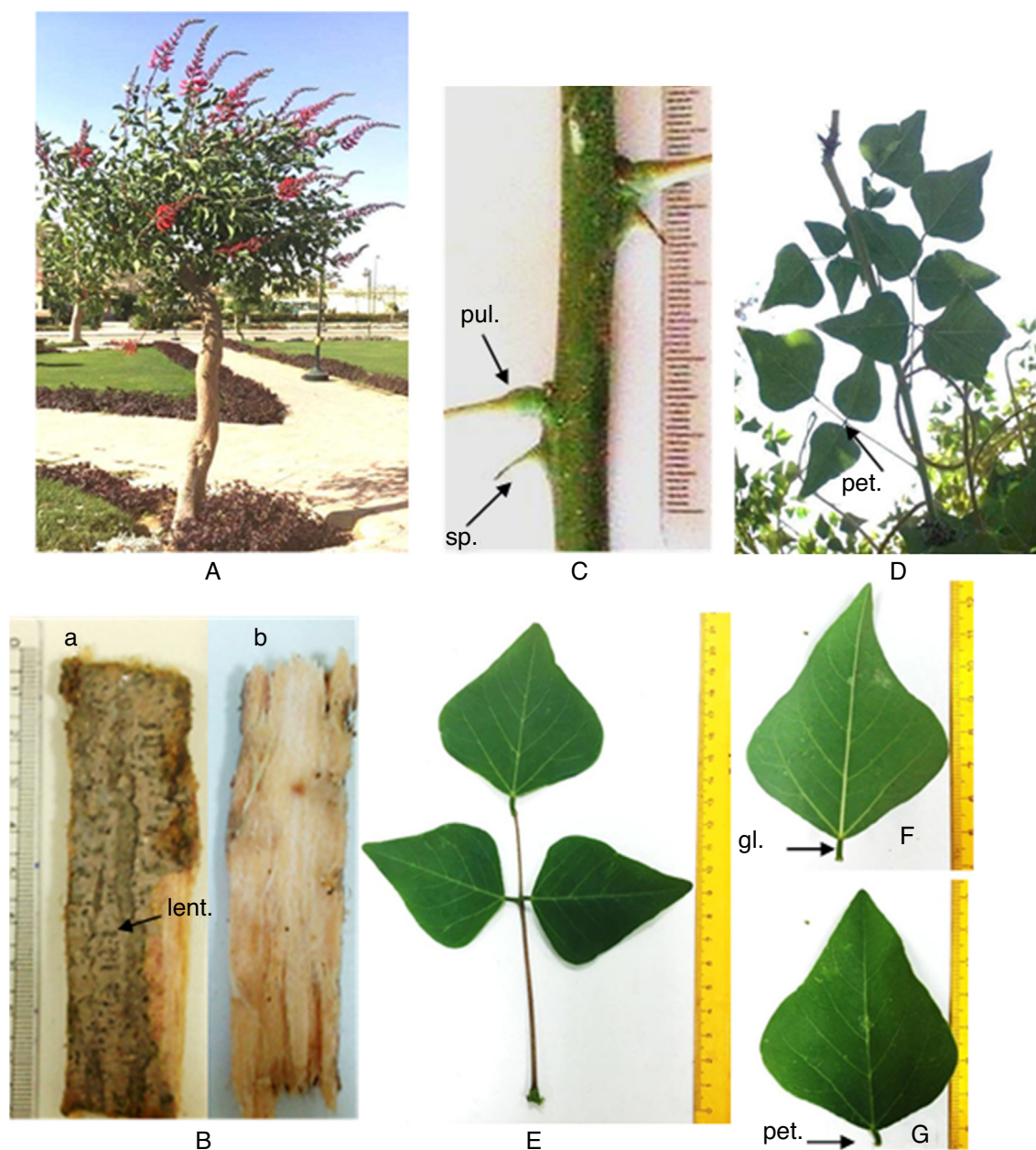
Fig. 1. *Erythrina × neillii* Mabberley & Lorence, Fabaceae (A–G). (A) Photograph of the plant ($X = 0.03$). (B) Bark ($X = 0.75$) a. Outer surface, b. Inner surface. (C) Branch showing spines and pulvinus ($X = 1.1$). (D) Branch showing alternate compound leaf ($X = 0.12$). (E) Imparipinnate leaf ($X = 0.31$). (F) Adaxial surface of the leaflet ($X = 0.42$). (G) Abaxial surface of the leaflet ($X = 0.42$). gl., gland; lent., lenticel; pet., petiolule; pul., pulvinus; sp., spines.

consists of thin cellulosic walled cells traversed by biseriate to multiseriate medullary rays. Medullary rays are formed of rectangular cells with pitted walls which are thin cellulosic in phloem and thick lignified in xylem. The cambium (Fig. 2B) is formed of 3–4 lines of tangentially elongated, cellulosic thin walled cells. The xylem (Fig. 2C) consists of lignified spiral, pitted and reticulate vessels. The wood fibers are fusiform with a wide lumen and acute to round apices. Wood parenchyma consists of rectangular elongated cells with pitted lignified walls.

The pith consists of round to polygonal parenchymatous cells having thin cellulosic walls and showing prisms of calcium oxalate and starch granules. Secretory cells or sacs with or without tanniferous content are present in the cortex, phloem and pith.

The powdered stem (Fig. 2C) is yellowish green in color, odorless and with slightly bitter taste. It is characterized by: fragments of epidermal cells showing paracytic and anomocytic stomata, glandular club shaped hair and non-glandular unicellular to multicellular two-armed hair, fragments of pericyclic fibers having thick lignified walls, narrow lumen and tapering acute apices forming crystal sheath, scattered prisms of calcium oxalate, amorphous contents and starch granules either free or in parenchyma cells. Fragments of lignified, spiral, pitted and annular xylem vessels, lignified wood fibers with wide lumen and acute to round apex and fragments of wood parenchyma and medullary rays are also observed.

The old stem

The old stem is more or less rounded in outline (Fig. 3A). It shows an outer dark brown band of cork (4–5 layers), phelloderm (4–6 layers) and primary cortex (4–8 layers) of collapsed
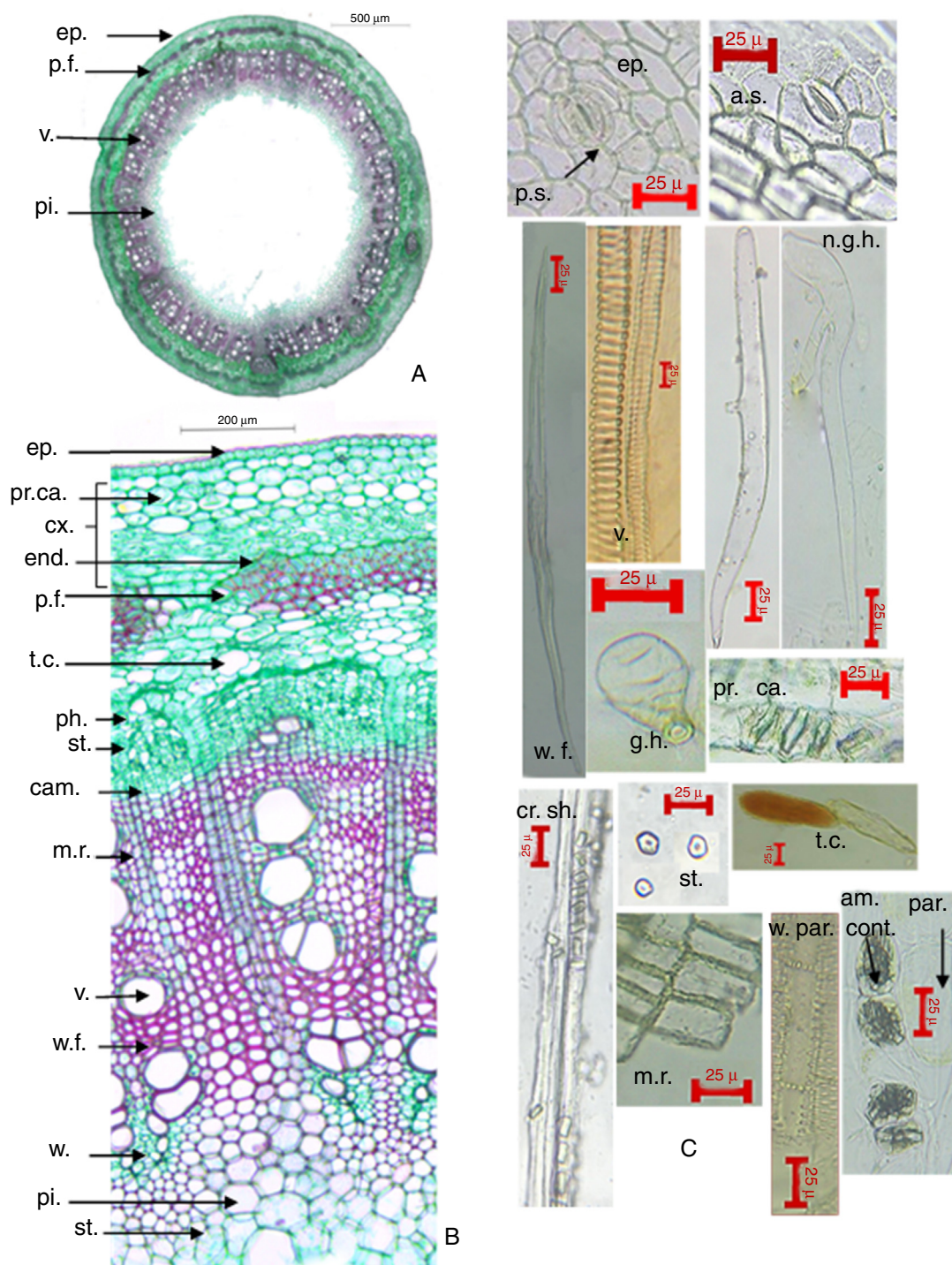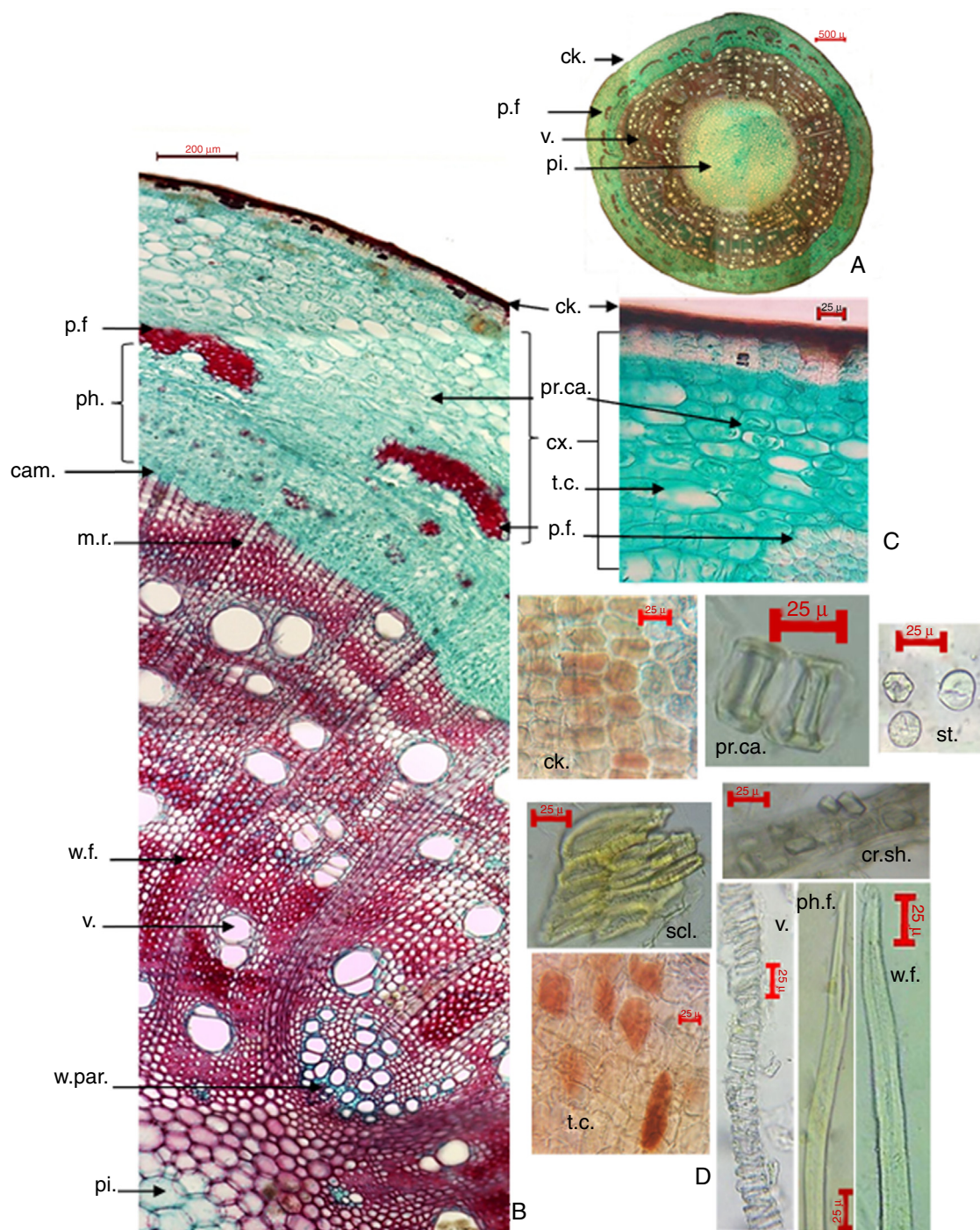
Fig. 2. Young stem of *Erythrina × neillii* Mabberley & Lorence, Fabaceae (A–C). (A) Low power view of the Young stem ($X=20$). (B) High power view of the young stem ($X=100$). (C) Powdered young stem. am. cont., amorphous content ($X=240$); a.s., anomocytic stomata ($X=400$); cam., cambium; cr.sh., crystal shealth ($X=200$); cx., cortex; end., endodermis; ep., epidermis ($X=400$); g.h., glandular hair ($X=480$); n.g.h., non-glandular hair ($X=260$); m.r., medullary rays ($X=320$); p.f., pericyclic fibers ($X=240$); p.s., paracytic stomata; par., parenchyma; ph., phloem; pi., pith; pr.ca., prism of calcium oxalate ($X=320$); st., starch ($X=240$); t.c., tannin cell ($X=120$); v., xylem vessel ($X=160$); w.f., wood fiber ($X=240$); w. par., wood parenchyma ($X=240$).

parenchyma (Fig. 3B and C). The pericycle shows the presence of groups of pericyclic fibers forming a crystal sheath. The vascular tissue is wider compared with the young stem while the pith is narrower. Secretory cells, calcium oxalate prisms and starch granules are distributed in the cortex and phloem tissue. Groups of sclereids are scattered in the pericycle and phloem. The sclereids are oval to isodiametric with thick lignified walls and narrow lumen.

The powdered stem (Fig. 3D) has a bitter astringent taste, odorless and yellowish brown in color. It is characterized by fragments of cork cells, polygonal, with thick lignified walls and yellowish brown content. Fragments of crystals sheath, scattered prisms of calcium oxalate, tannin cells or sacs, starch granules (either free or in parenchyma cells), sclereids, wood elements and phloem fibers are also identified. Phloem fibers are elongated, fusiform with narrow lumen, thick lignified walls and acute to forked apex. Pericyclic

Fig. 3. Old stem of *Erythrina × neillii* Mabberley & Lorence, Fabaceae (A–D). (A) Low power view of the old stem (*X* = 10). (B) High power view of the old stem (*X* = 65). (C) High power view of the old stem in the cortex region (*X* = 160). (D) Powdered old stem. cam., cambium; ck., cork (*X* = 200); cr.sh., crystal shealth (*X* = 200); cx., cortex; m.r., medullary ray; p.f., pericyclic fibers; ph., phloem; ph.f., phloem fiber (*X* = 280); pi., pith; pr.ca., prism of calcium oxalate (*X* = 400); scl., sclereids (*X* = 200); st., starch (*X* = 280); t.c., tannin cell (*X* = 120); v., xylem vessel (*X* = 200); w.f., wood fiber (*X* = 280); w.p., wood parenchyma.

fibers have thick lignified walls, narrow lumen and tapering acute apices

The stem bark

The transverse section (Fig. 4A) of the stem bark shows the cork arranged in radial and tangential layers followed by phelloderm and primary cortex showing secretory cavities filled with brown contents. Pericycle is formed of parenchymatous cells interrupted by groups of fibers and accompanied by sclereids. The phloem is traversed by medullary rays, tangential bands of bast fibers and groups of sclereids.

Cork cells (Fig. 4A and B) are brown polygonal with straight thick lignified anticlinal walls. The cork cambium is followed by the secondary cortex which is formed of 5–7 layers of slightly thick-walled tangentially elongated cellulosic parenchymatous
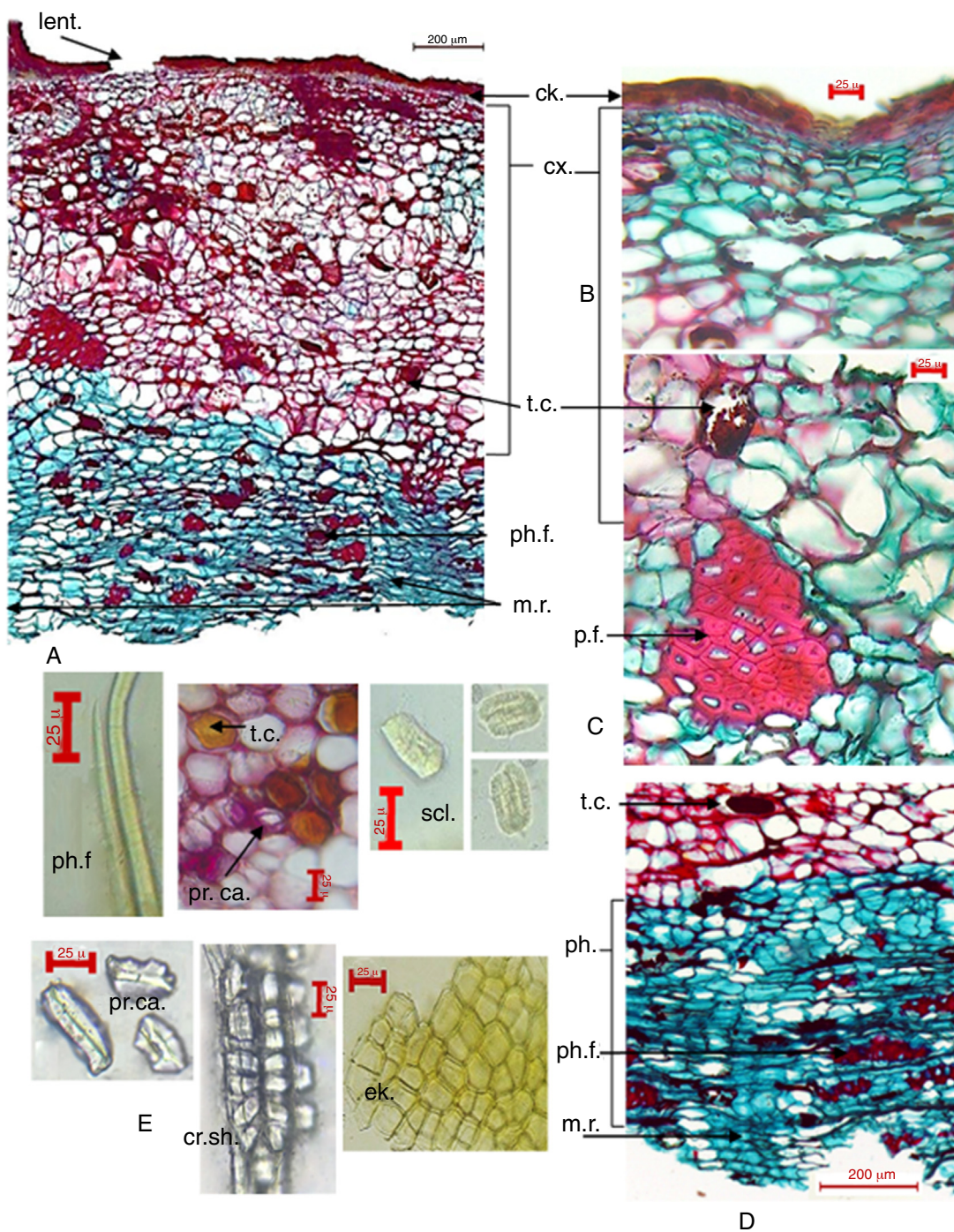
Fig. 4. Stem bark of *Erythrina × neillii* Mabberley & Lorence, Fabaceae (A–E). (A) Cross-sectional view of the stem bark (X = 50). (B)–(D) High power view of the stem bark (X = 200–200–70). E. Powdered stem bark. ck., cork (X = 200); cr.sh., crystal shealth (X = 200); cx., cortex; lent., lenticels; m.r., medullary ray; p.f., pericyclic fibers; ph., phloem; ph.f., phloem fibers (X = 400); pr.ca., prism of calcium oxalate (X = 240); scl., sclereids (X = 360); t.c., tannin cells (X = 200).

cells, followed by rounded parenchyma cells of primary cortex. The pericycle (Fig. 4B and C) is parenchymatous interrupted by groups of lignified pericyclic fibers and sclereids. The sclereids are oval to isodiametric with thick lignified walls and narrow lumen. The phloem consists of primary and secondary phloem traversed by bi-multiseriate medullary rays and tangential groups of lignified phloem fibers. The medullary rays (Fig. 4D) are formed of rectangular, radially elongated and rounded parenchymatous cells. Tannin cells, starch granules and prisms of calcium oxalate are scattered in the tissues of cortex, pericycle and phloem.

The powdered stem bark (Fig. 4E) has a bitter astringent taste, brown color and characterized by: fragments of cork cells, phloem fibers, crystal sheath, sclereids, and secretory tannin cells, prisms of calcium oxalate and starch granules either free or in parenchymatous cells.

The leaflet

Transverse section of the leaflet (Fig. 5A) shows isobilateral structure, with discontinuous palisade in the midrib region. The midrib shows crescent shaped large vascular bundle and small
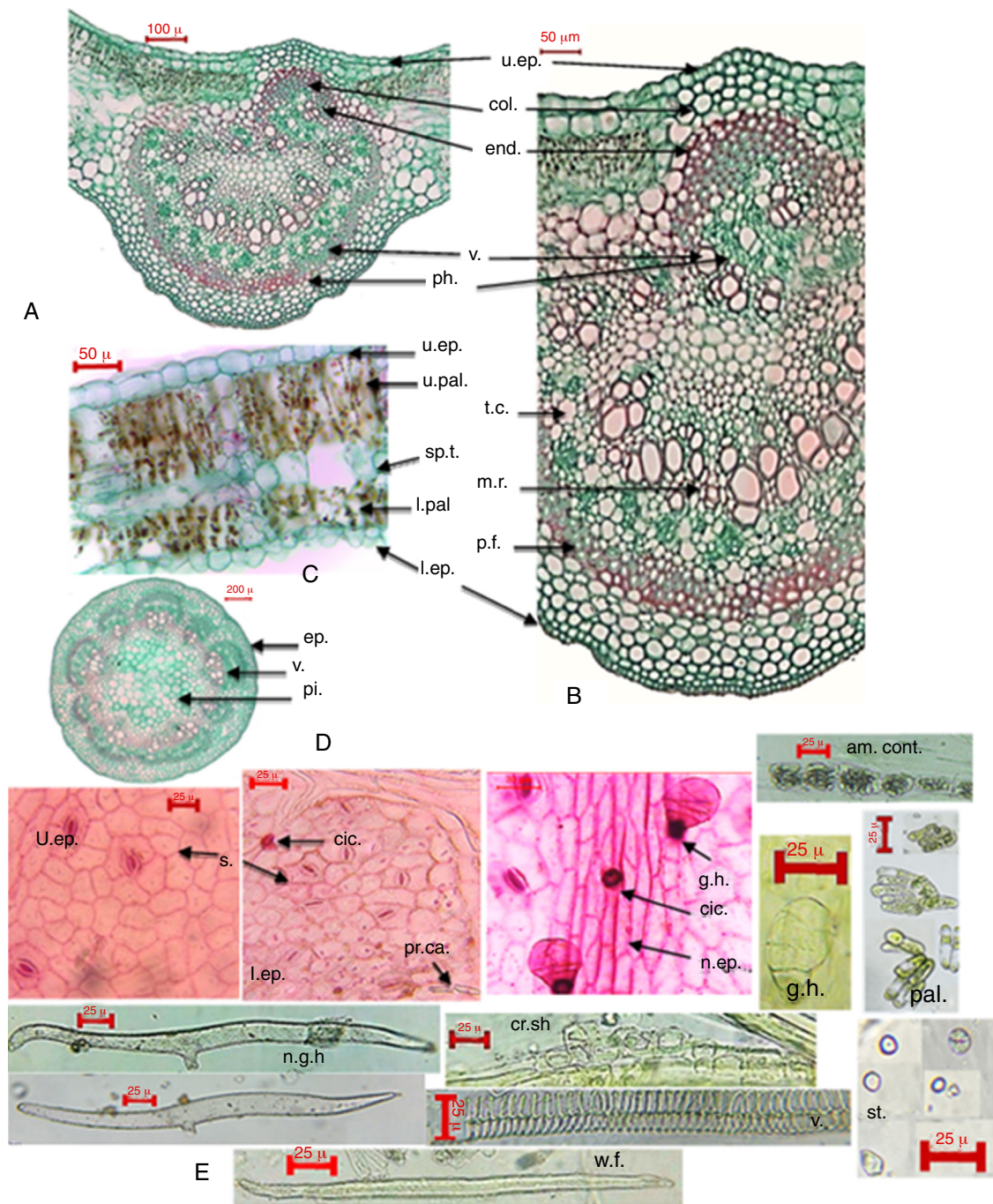
Fig. 5. Leaf of *Erythrina* × *neillii* Mabberley & Lorence, Fabaceae (A–E). (A) Low power view of the lamina (X = 70). (B) High power view of the midrib (X = 100). (C) High power view of the lamina (X = 140). (D) Low power view of the petiole (X = 25). (E) Powdered leaf. am. cont., amorphous content; cic., cicatrix (X = 160); col., collenchyma; cr.sh., crystal shealth (X = 160); end., endodermis; g.h., glandular hair (X = 400); l.ep., lower epidermis (X = 160); l.pal., lower palisades; m.r., medullary rays; n. ep., neural epidermis (X = 233); n.g.h., non-glandular hair (X = 160); pal., palisades (X = 160); p.f., pericyclic fibers; ph., phloem; pi., pith; pr.ca., prism of calcium oxalate; s., stomata; sp.t., spongy tissue; st., starch (X = 320); t.c., tannin cells; u.ep., upper epidermis (X = 120); u.pal., upper palisade; v., xylem vessel (X = 200); w.f., wood fiber (X = 280).

Table 1
Total number of amplicons as revealed by RAPD markers among the five accessions.

PRIMER	E × neilli	E. indica	E. speciosa	E. variegata	E. japonica
OPA-11	4	4	3	3	2
OPA-10	3	4	3	5	7
OPB-06	4	2	3	3	3
OPB-07	4	3	3	3	3
OPD-15	5	2	3	3	3
OPD-14	2	2	6	3	3
OPC-01	3	2	3	3	3

inverted one. The pericycle is sclerenchymatous and the endodermis is distinct. The upper and lower epidermises (Fig. 5B and C) are covered by smooth cuticle and shows paracytic and rarely anomocytic stomata. The epidermis surface in the abaxial surface is papillosed, with beaded anticlinal wall, more frequent stomata and less frequent trichomes, while neural epidermal cells are elongated and rectangular. The mesophyll is isobilateral with discontinuous palisade at the midrib. The palisade is formed of 2–3 layers on the upper surface and 1–2 short layers in the lower one. The spongy tissue is formed of 3–5 layers of thin-walled, irregular parenchyma
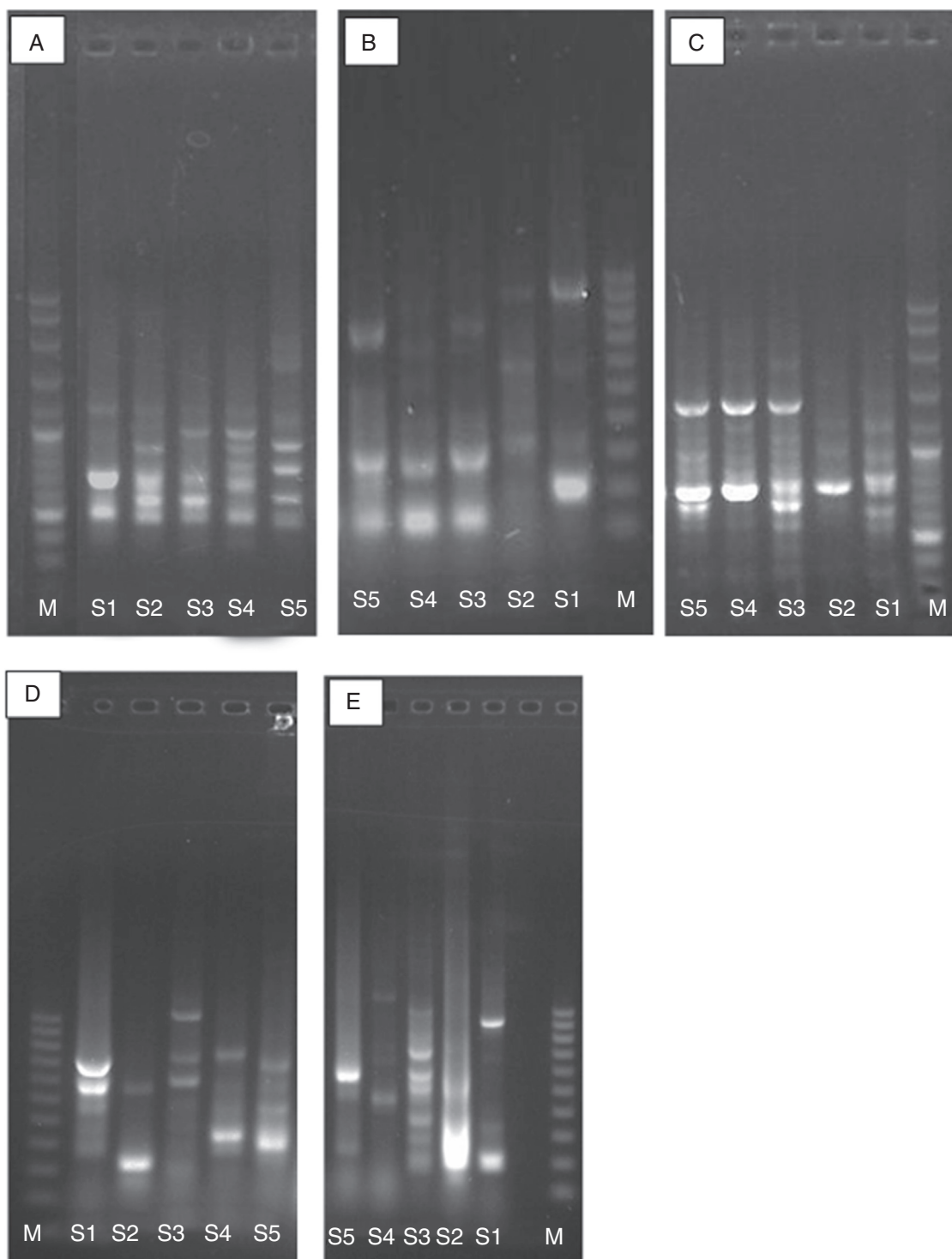
Fig. 6. (A–E) RAPD gel comparing different *Erythrina*. (A) RAPD gel OPA-10. (B) OPB-07. (C) OPD-14. (D) OPB-6. (E) OPD-15; M, Marker; S1, *Erythrina* × *neilli*; S2, *E. indica*; S3, *E. speciosa*; S4, *E. variegata* and S5, *E. japonica*.

with wide intercellular spaces, containing scattered prismatic crystals of calcium oxalate, tannin cells and few simple starch granules. The cortical tissue of the midrib is more prominent on the lower surface. It consists of two layers of collenchyma followed by 4–5 layers of parenchymatous cells. The vascular bundle is collateral surrounded by sclerenchymatous pericycle, consisting of lignified fibers forming an arc below the large vascular bundle and a patch above the inverted small vascular bundle and forming a crystal sheath. Prismatic crystals of calcium oxalate, simple starch

granules and tanniferous cells are scattered in the cortical tissue and phloem.

The petiole

The petiole is more or less rounded (Fig. 5D), similar to the young stem except for the absence of a secondary thickening and the less lignified elements. It is formed of an outer epidermis, 2–3 layers of collenchyma and followed by 4–5 layers of parenchymatous
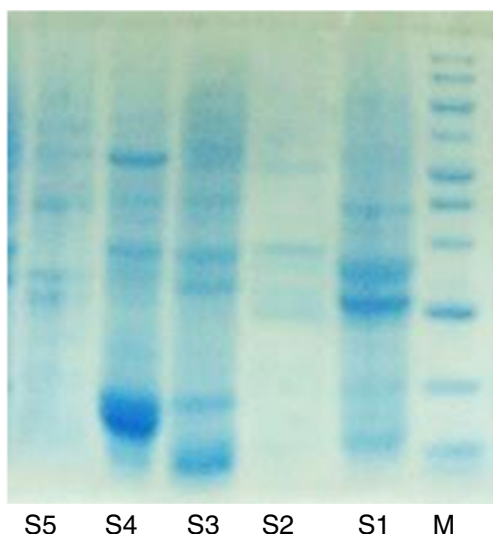
Fig. 7. SDS protein gel comparing different types of *Erythrina*. M, Marker; S1, *Erythrina* × *neilli*; S2, *E. indica*; S3, *E. speciosa*; S4, *E. variegata* and S5, *E. japonica*.

cells. Prisms of calcium oxalate and starch granules are scattered through the cortical tissue. Inside the central cylinder, there are approximately 9–10 collateral vascular bundles with fiber caps on the phloem. The powdered leaf (Fig. 5E) is dark green in color with astringent taste, characterized by: fragments of upper and lower epidermis showing paracytic and anomocytic stomata, glandular and non-glandular hair, unicellular or multicellular hair with unequal two arms. Fragments of palisade cells, parenchyma cells containing amorphous brown content, starch granules and prisms of calcium oxalate free or in cells, xylem vessels and crystal sheath are also identified.

Randomly Amplified Polymorphic DNA (RAPD) analysis

Seven decamer RAPD primers were screened with the DNA of the five *Erythrina* accessions showing reproducible and scorable RAPD profiles with a number of amplified DNA fragments ranging from 14 to 22. A maximum number of 22 amplicons were amplified with primer OPA-10, while the minimum number of fragments (14) was amplified with primer OPC-1 (Table 1, Fig. 6A–E).

The RAPD results revealed very high intra-varietal polymorphism. Regarding the SDS-PAGE (Fig. 7), all the samples are sharing the same bands in 55, KD, sample 4 is missing the upper bands and yet it is revealed in a very low concentration in both samples 2, 3 but all the plant samples have the lower bands as the control.

Discussion

The exact identification of plant species showing similarities represents a real obstacle. This is aggravated in cases of hybrids. Morphological and anatomical descriptions allow to distinguish among the closely related species and resolve to a certain extent this complexity (Da Silva et al., 2013).

Anatomical characters including secretory structures, types of stomata, trichomes and calcium oxalate that are characteristic for erythrina genus were reported by Metcalfe and Chalk, (1950) and helped in accurate identification of *Erythrina*×*neilli*. Isobilateral mesophyll is observed in agreement with *E. cristagalli*, while *E. velutina* and *E.falcata* show dorsiventral mesophyll (Gratieri-Sossela, 2005; Almeida, 2010; Almeida, 2011). Concerning the types

of stomata found in *Erythrina* × *neilli*, (paracytic and anomocytic), there is a certain similarity to other *Erythrina* species where paracytic stomata represents a common character as present in *E. velutina* (Da Silva et al., 2013) *E. speciosa*, and *E. falcata* (Almeida, 2010; Almeida, 2011).

Although anatomical studies still represent an important tool for the identification of different species, molecular markers are efficient tools for authentication of plant materials through DNA based techniques (Parveen et al., 2016). Although *Erythrina* × *neilli*, showed some similarities to the other species in its morphological characters, however, at the molecular level, differences are present. In the present investigation, RAPD was employed to assess the genetic polymorphism among five *Erythrina* species. It was capable to identify very high intra-varietal polymorphism among the five species which denotes the necessity for in-depth study of the molecular differences between the studied species.

Authors' contribution

SKG contributed in collecting and running the laboratory work. ROB, supervised the laboratory work, and contributed in writing the manuscript. AME, and TSE contributed in designing the study, critical analysis of data, supervised the laboratory work. HME performed the molecular characterization. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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