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Lavandula spp. diversity assessment by molecular markers as a tool for growers

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Lavender is one of the most important medicinal plants. The quality and the therapeutic properties of the essential oil of lavender are determined by the quantity and the biological activity of the individual components. Different lavender varieties often have a characteristic profile, differing in their individual substances, with small differences affecting the aroma and properties of the essential oil. Genetic variation between species and cultivars, as well as environmental conditions, nutrition, season, and type of tissue, all influence the chemical composition of lavender essential oil. The collection of twelve genotypes of *Lavandula* spp., which includes species of *L. angustifolia* (Mill.), and *Lavandula* × *intermedia* Emeric ex Loisel. were grown in the locality of Malé Leváre (Slovakia). These species, without further specification of varieties, were screened for DNA polymorphism by random amplified polymorphic DNA (RAPD) markers. Leaf and flower samples were analyzed using 4 universal random decamers. Two decamers (OPB 11 and OPB 18) provided efficient and reproducible amplification profiles. Individual genotypes and leaf and flower samples were characterized using DNA fingerprint cards containing digital electrophoretic profiles generated by random decamer primers and the corresponding image of the lavender genotype. This low-cost and effective approach to genetic diversity screening can provide useful documentation for lavender growers and additional genotype specifications.

Keywords: Lavender, RAPD, DNA fingerprinting, DNA polymorphism, genetic diversity

Introduction

One of the most commercially exploited species is *Lavandula angustifolia* Mill., which originated in the Mediterranean region, but today the cultivation of this species and its cultivars is widespread all over the world, including Slovakia. Species belonging to the genus Lavender are rich in secondary metabolites such as phenolic acids, flavonoids, coumarins, terpenes,

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and tannins (Hawrył et al., 2019). Lavender is one of the most important medicinal plants, and due to its calming effects, this species is popularly used as part of adjunctive treatment for anxiety conditions, to relieve psychological stress, or to induce sleep (Ghavami et al., 2022). Lavender is mainly cultivated for the production of lavender essential oil, which is used in perfumes, cosmetics, gastronomy, and aromatherapy (Lis-Balchin, 2002). The genus Lavender includes approximately 47 species, dozens of subspecies, and hundreds of hybrids (Prusinowska and Śmigielski, 2014). The most important and economically valuable species include: *L. angustifolia* Mill., *L. latifolia* Medik., *L. stoechas* L., and *L. intermedia* Emeric ex Loisel.

Different lavender species often have a characteristic profile, differing in the representation of individual compounds, with even small differences affecting the aroma and properties of the essential oil (Gonçalves and Romano, 2013; Demasi et al., 2018). Thus, the chemical composition of lavender essential oil is largely influenced by genetic variation among species and cultivars, but also by external factors such as, for example, temperature conditions, water quantity, altitude and geographical location, fertilizers, season, or the type of weed from which it was obtained (Lis-Balchin, 2002; Demissie et al., 2011; Hassiotis et al., 2014).

The morphological, agronomic, and other characteristics required to classify and identify plant genotypes within species are effectively complemented by molecular markers. The importance and usefulness of these characteristics are considerable, both for research and breeding as well as for practical use (Gálová et al., 2013, 2016). Studying and comparing the molecular information of individual organisms involves searching for DNA polymorphisms (Malik et al., 2017). Although random amplified polymorphic DNA (RAPD) has several limitations, it is still an effective tool for DNA polymorphism detection. It is low-cost method, detecting a large number of polymorphic loci and revealing genomic variation in coding and non-coding regions of the genome (Zhang et al., 2007; Hnia et al., 2013). RAPD uses a polymerase chain reaction to amplify random DNA fragments using short oligonucleotides, most commonly decamers. The nucleotide order of the primers is random, with 50-80% guanine content and cytosine. After propagation, the fragments are resolved in an agarose gel. In the PCR reaction, a single random primer species binds to genomic DNA at two different positions on the complementary strands of the template DNA. If these binding sites are within the amplifiable range, the specific DNA fragment is amplified. The presence of RAPD fragments in the electrophoretic profile corresponds to the dominant allele and the absence of the recessive allele. Amplified fragments range in size from 0.5–5.0 kb (Bežo et al., 2015).

The work aimed to apply a universal random molecular marker RAPD to obtain genotype-specific DNA fingerprints of lavender genotypes, specifically for leaves and flowers samples, and design for each variety a "DNA fingerprint card" documentation providing the grower with a closer specification of cultivated genotypes.

Material and methodology

A number of different genotypes of lavender plants have been made available by the lavender grower and owner of the company "Levanduland, Ltd." in the town of Malé Leváre (https://levanduland.sk/). Except for the area of 1 hectare, where are cultivated 13 000 seedlings of *L. angustifolia* Mill., the grower collected various species of lavender cultivated on a small experimental area. This collection includes species of *L. angustifolia*, and *Lavandula* × *intermedia* (Figure 1), without further variety specification.

Sampling

Three plants per genotype were collected, marked by the position code (designation of the line/the order of the plant in the row) for matching the results to individual genotypes (Figure 1). At the time of sampling, a single genotype (Figure 2 F) had not flowered yet, so the analyses come only from leaf samples. Immediately after collection, the samples were placed in a labeled bag and stored in a portable refrigerated container containing the frozen plates. Upon arrival at the laboratory, photo documentation (Figure 2) was made with close-ups of the flowers, and the plants were briefly stored at 20 °C.

Genomic DNA isolation

Genomic DNA from leaves and flowers of individual genotypes was isolated by NucleoSpin Plant II DNA isolation Kit (Macherey Nagel™) according to the manufacturer's instructions, with subsequent DNA quality and quantity control by Nanophotometer (Implen P360). Since the purity of the DNA obtained was not satisfactory due to the high abundance of secondary metabolites, the samples had to be purified.

Figure 1 View of the experimental area with different genotypes of lavender in the company "Levanduland, Ltd."

DNA purification

Samples were purified by adding 1/10 of a sample volume of 3 mol.dm^{-3} NaOAc pH 5.2 and 2.5 sample volumes of 100% EtOH. Samples were incubated overnight at -20 °C followed by centrifugation at 12000 rpm, for 20 minutes. After pipetting the supernatant, 400 µl of 70% EtOH was added to the DNA pellet and then centrifuged for 5 minutes at 12000 rpm. The precipitate was dissolved in 30 µl of ultrapure H2O.

RAPD-PCR Assay

RAPD primers sequences originated from a decameric oligonucleotides database: OPB-05 (5´ TGC GCC CTTC 3´), OPB-11 (5´ GTA GAC CCGT 3´), OPB-18 (5´ CCA CAG CAGT 3´) and OPB-20 (5´ GGA CCC TTAC 3´) were applied on leaf and flower samples of twelve lavender genotypes. Solis Dynazyme chemicals were used for RAPD-PCR assay realized by C1000 Thermal Cycler (BIO-RAD) as follows: 1 × Buffer B (0.8 mol.dm^{-3} Tris-HCl, 0.2 mol.dm^{-3} $(NH_4)_2SO_4$), 2.5 mmol.dm^{-3} $MgCl_2$, 0.2 mmol.dm^{-3} dNTP, 2U FIREPol DNA polymerase, 0.4 µmol.dm^{-3} primer and 30 ng of DNA. The amplification of each sample was repeated twice. The amplification protocol was as follows: initial denaturation at 94 °C for 2 minutes, followed by 45 cycles of denaturation at 94 °C for 1 minute, annealing at 36 °C for 1 minute, polymerization at 72 °C for 2 minutes, and the final polymerization at 72 °C for 7 minutes.

Electrophoresis of amplified products

The amplification assay was followed by electrophoresis analysis on a 1.5% agarose gel with a 1 kb size marker (Bioron, ready to use). A loading dye (6 × loading dye, Invitrogen) was added to the PCR samples and 10 µl of the sample was loaded onto the gel. Electrophoresis was performed at a constant voltage of 100 V for 45 minutes.

Statistical analysis

Amplification of each sample followed by electrophoretic separation of DNA fragments was repeated twice. The electrophoreograms were recorded by GeneSnap software (Syngene). The amplification results were processed based on digital recordings of electrophoreograms by GeneTool Analysis software (Syngene).

Figure 2 Lavender genotypes collected from the experimental area: A–L represent individual lavender genotypes

Results and discussion

Genomic DNA polymorphism of twelve different lavender genotypes of various species (*L. angustifolia*, and *Lavandula* × *intermedia*), was analyzed by four RAPD primers (OPB-05, OPB-11, OPB-18, and OPB-20). Two of them (OPB5 and OPB 20) did not provide efficient amplification profiles for all samples analyzed. DNA polymorphism of analyzed samples was observed with primers OPB-11 (5´ GTA GAC CCGT 3´) and OPB-18 (5´ CCA CAG CAGT 3´). By the primer, OPB-11 have amplified 127 DNA fragments in total, of which 64 in leaf samples and 63 in flower ones. For the primer OPB-18 has observed 151 DNA fragments in total, out of which 73 were for leaf and 78 were for flower samples.

The main output of this study was to provide growers with documentation in the form of 'DNA fingerprint cards' for each lavender genotype grown in the trial area. DNA fingerprint cards generated by RAPD marker OPB-11 are presented in Figures 3–5 and by marker OPB-18 in Figures 6–8. For each genotype, cards are provided separately for leaf and flower samples.

There are different types of molecular markers. They may differ in a number of ways – such as their technical requirements; time and price complexity (Žiarovská et al., 2011, 2016; Gálová et al., 2013, 2016). The presence of polymorphisms between individuals will lead to a different pattern of markers after electrophoresis has been performed. These patterns are comparable to a "fingerprint", therefore these techniques are

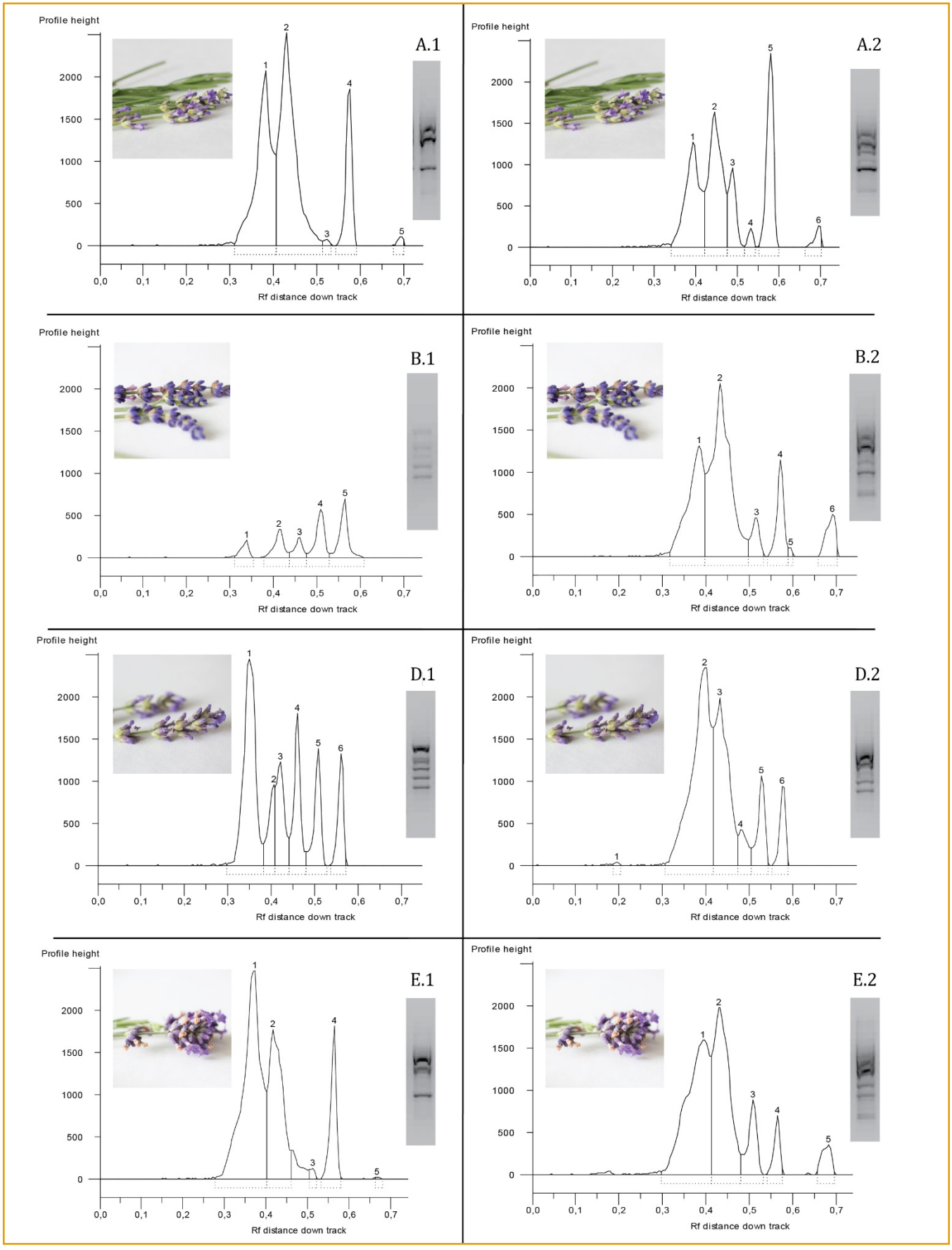

Figure 3 DNA fingerprint cards generated by OPB-11
A–D represent lavender genotypes; 1 – leaf sample; 2 – flower sample

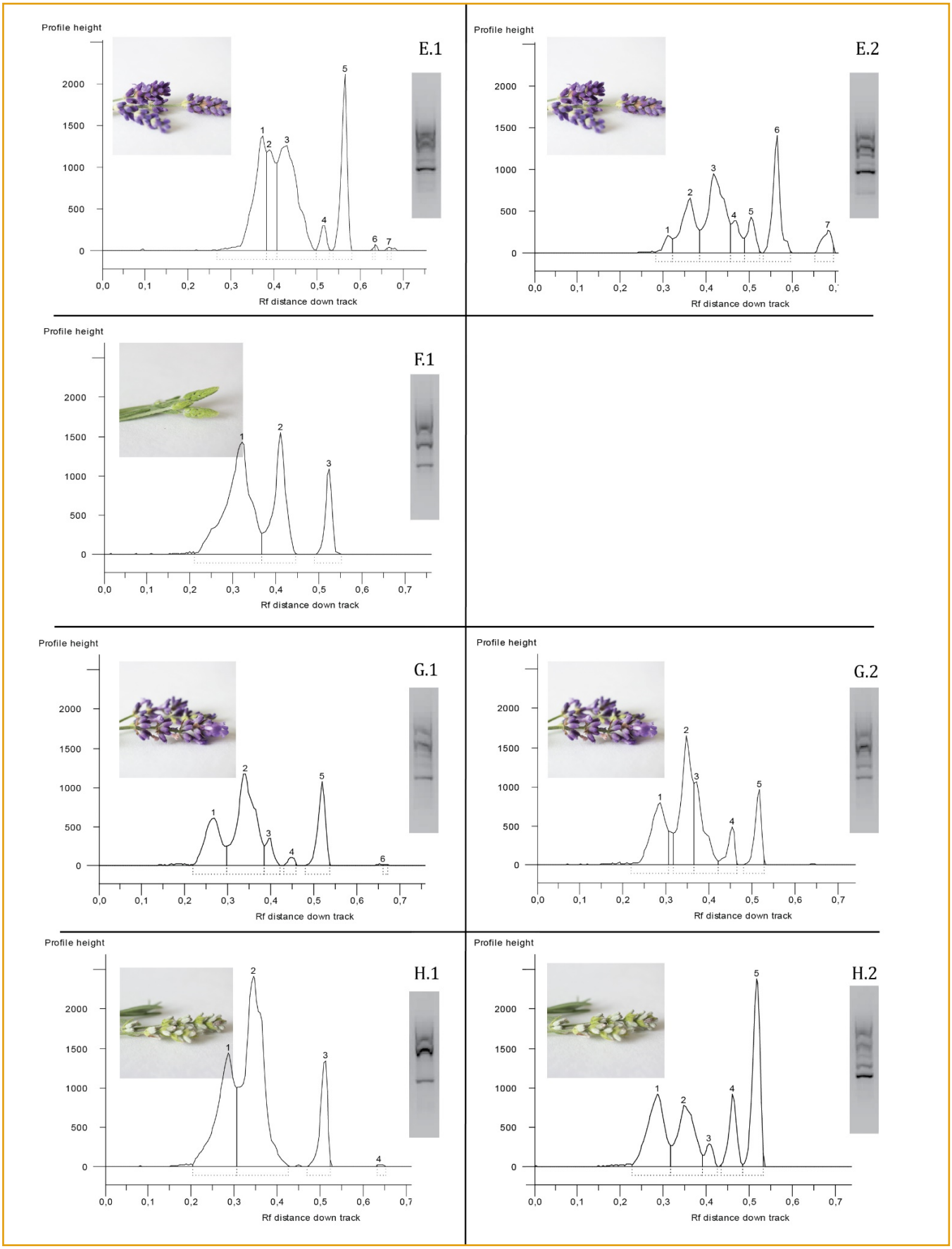

Figure 4 DNA fingerprint cards generated by OPB-11
E–H represent lavender genotypes. 1 – leaf sample; 2 – flower sample. Genotype F was the only one not yet flowering at the time of sampling

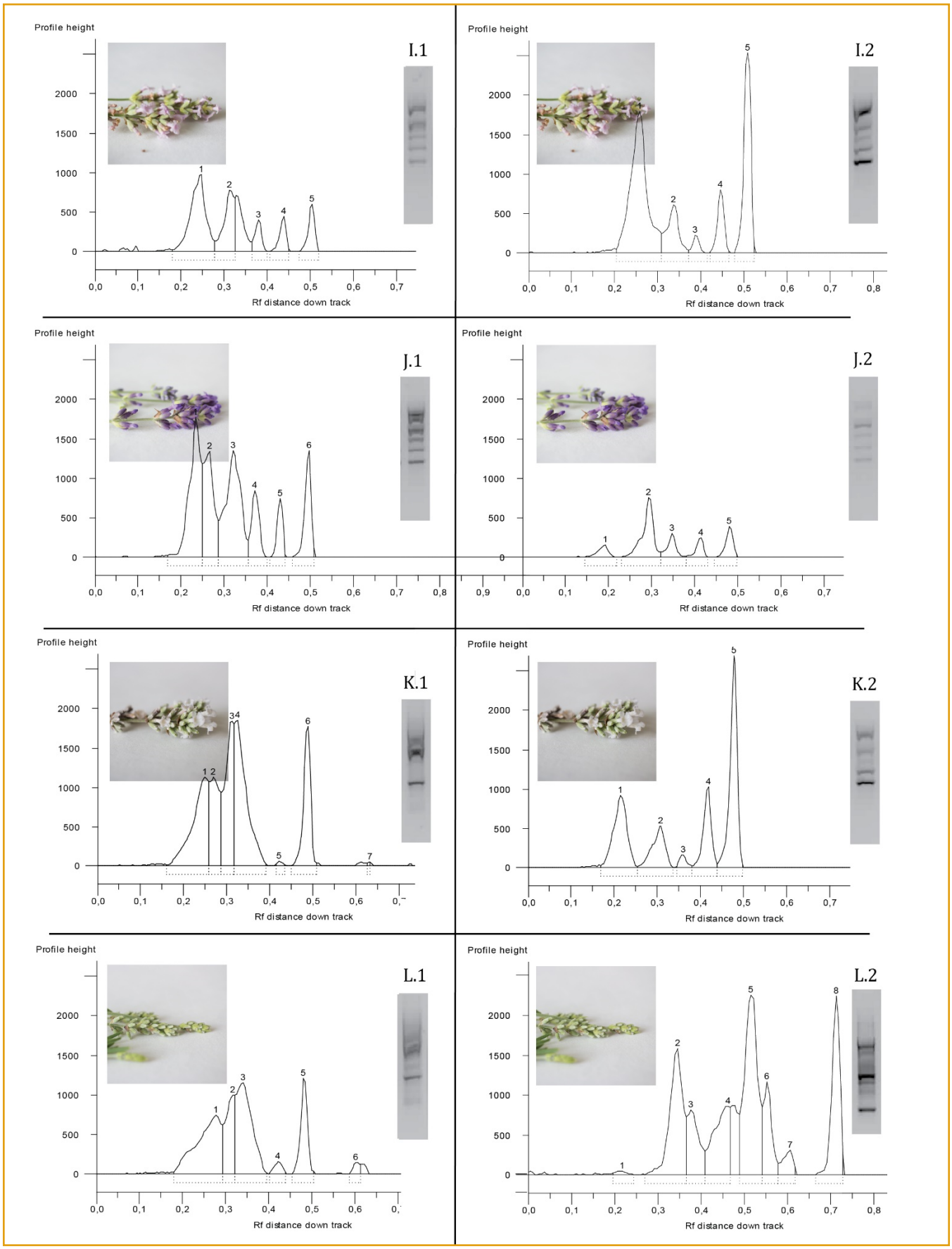

Figure 5 DNA fingerprint cards generated by OPB-11
I–L represent lavender genotypes. 1 – leaf sample; 2 – flower sample

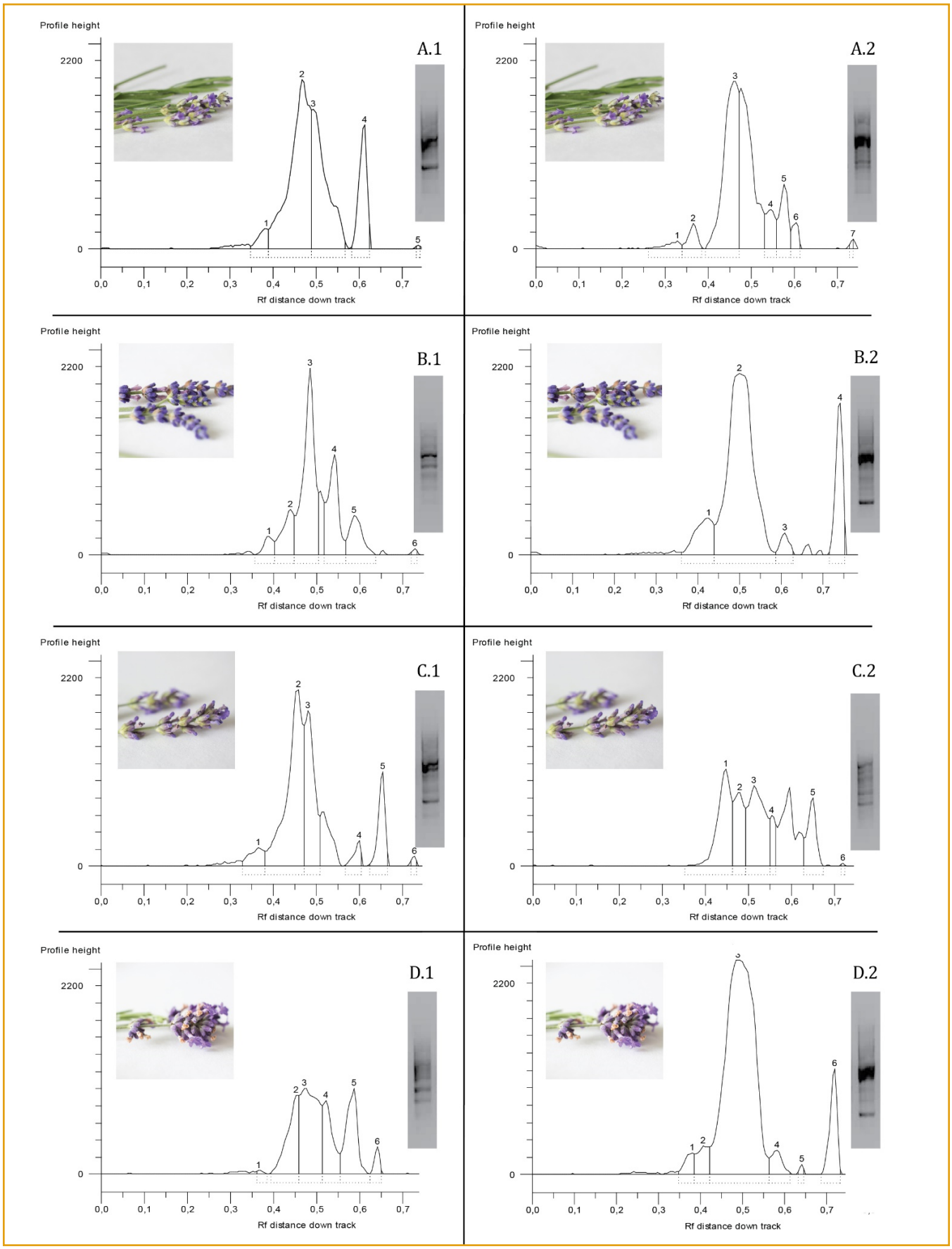

Figure 6 DNA fingerprint cards generated by OPB-18
A–D represent lavender genotypes. 1 – leaf sample; 2 – flower sample

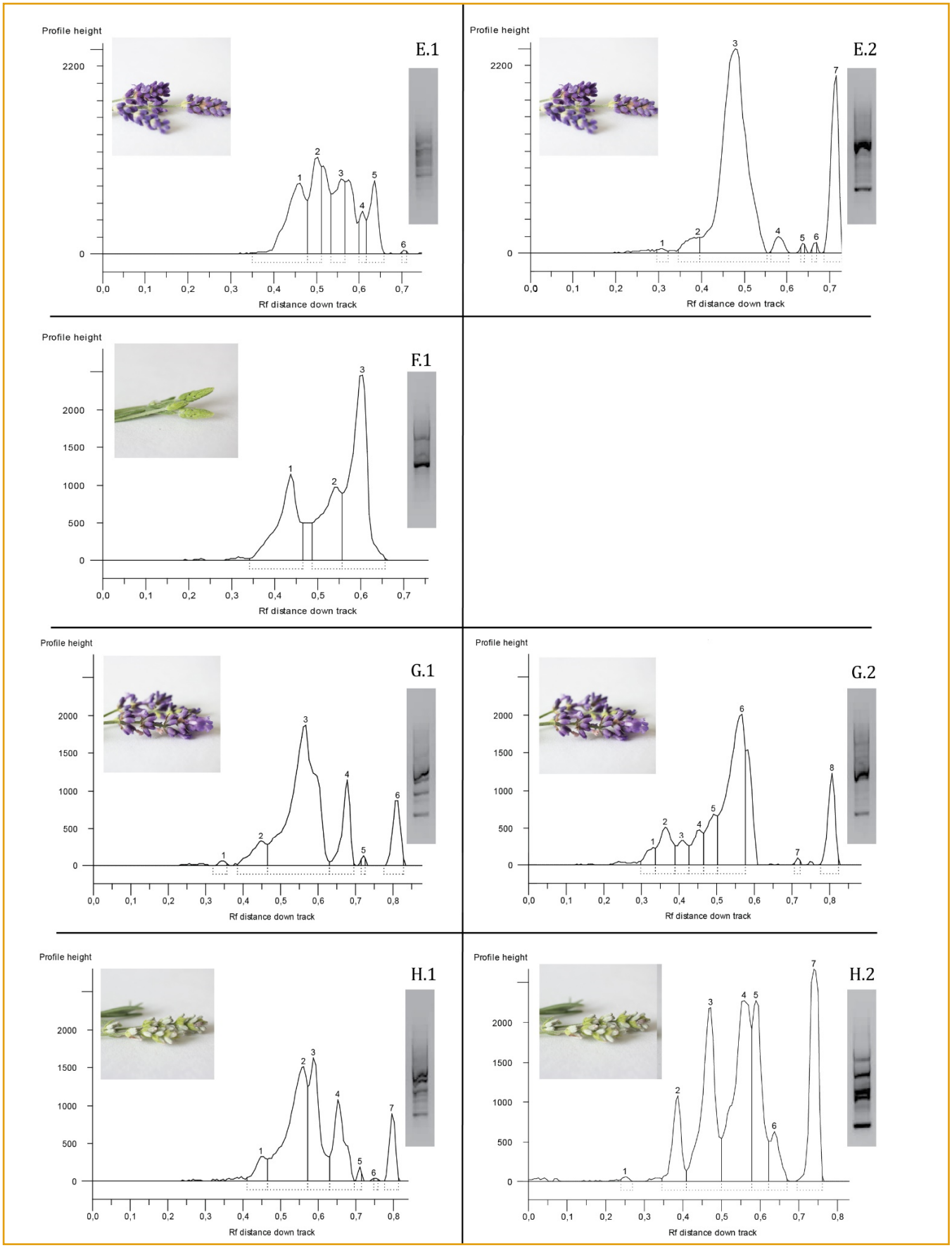

Figure 7 DNA fingerprint cards generated by OPB-18
E–H represent lavender genotypes. 1 – leaf sample; 2 – flower sample. Genotype F was the only one not yet flowering at the time of sampling

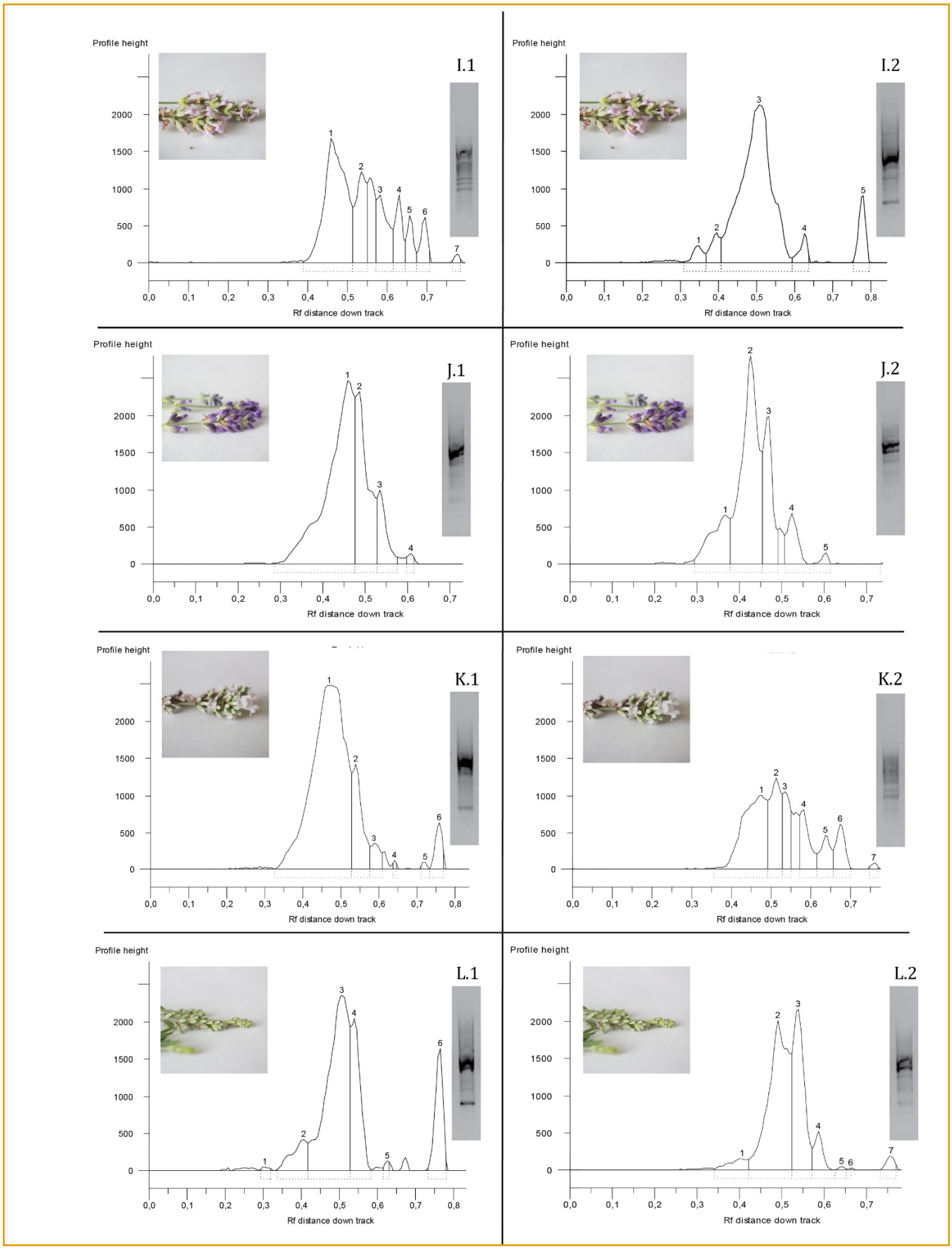

Figure 8 DNA fingerprint cards generated by OPB-18
I–L represent lavender genotypes. 1 – leaf sample; 2 – flower sample

sometimes referred to as fingerprinting techniques. The DNA polymorphisms between the individuals tested are revealed by these patterns (Poczai et al., 2013; Batley, 2015).

However, the quality of the isolated genomic DNA is also crucial to ensure efficient amplification. Emphasis is placed on the removal of residual secondary metabolites (Bulavin et al., 2020), as evidenced by our DNA isolation and purification procedure. For RAPD amplification was successful even isolation from dried leaves using the CTAB protocol (Ștefan et al., 2019).

The genetic diversity of seven *Lavandula* multifida L. populations from three bioclimates in Tunisia was estimated by RAPDs and allozymes (Hnia et al., 2017). By seven random decamer primers were amplified 97 RAPD markers, while in our case we observed a higher number of RAPD markers by one primer (127 by OPB-11 and 151 by OPB 18) from 12 samples. The effectiveness and sensitivity of RAPD markers are confirmed by the fact that relatively higher polymorphism was found in populations from the lower semi-arid bioclimate (Hnia et al., 2017).

The analysis of homology among 10 introduced lavender species was performed by RAPD. Fifteenth selected primers amplified 364 bands and the average number of bands amplified by a primer was 20.8 (Yanling et al., 2007). The collection of genotypes included species *L. angustifolia*, *L.* × *intermedia*, *L. stoechas*, *L. stoechas pedunculata*, *L. dentata* and *L. lannata* × *L. dentata*.

A similar study was performed with different populations of dentate lavender collected in the Algerian littoral (Gadouche et al., 2019). Despite the fact that the populations were sampled in different ecoregions, the dendrogram obtained by cluster analysis showed two clusters with a similarity index higher than 68%, demonstrating that the populations are genetically close despite their geographical distance.

Despite the sequence random origin of RAPD primers, not all types of decamers provide successful amplification, which has been demonstrated in our and other studies. We tested four random decametres out of which only two provided effective and reproducible amplification. To determine the intra- and interspecific genetic diversity of two *Lavandula* species: *L. angustifolia* Mill. (7 different varieties) and *L. stoechas* L., 14 RAPD primers were evaluated, of which 4 were selected according to the number of amplified polymorphic sites (Ștefan et al., 2019).

The rationale for the use of RAPD molecular markers for Lamiaceae species is supported by several current studies (Chowdhury et al., 2017; Ahmed and Al-Sodany, 2019; Saha et al., 2020; Sunar et al., 2020; Zhend et al., 2021; Ahmed et al., 2022).

Conclusions

This study aimed to provide an effective and low-cost approach for genetic diversity assessment useful for lavender growers to characterize individual genotypes. Obviously, a large-scale experiment applying a larger number of tested RAPD markers would be needed to complete the DNA fingerprinting identification cards. However, our analyses confirmed the suitability of this type of marker for such a complementary service for growers and practical use.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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