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Composition and stereochemistry of ephedrine alkaloids accumulation in *Ephedra sinica* Stapf

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ABSTRACT

Ephedra sinica Stapf (Ephedraceae) is a widely used Chinese medicinal plant (Chinese name: *Ma Huang*). The main active constituents of *E. sinica* are the unique and taxonomically restricted adrenergic agonists phenylpropylamino alkaloids, also known as ephedrine alkaloids: (1*R*,2*S*)-norephedrine (1*S*,2*S*)-norpseudoephedrine, (1*R*,2*S*)-ephedrine, (1*S*,2*S*)-pseudoephedrine, (1*R*,2*S*)-*N*-methylephedrine and (1*S*,2*S*)-*N*-methylpseudoephedrine. GC–MS analysis of freshly picked young *E. sinica* stems enabled the detection of 1-phenylpropane-1,2-dione and (*S*)-cathinone, the first two putative committed biosynthetic precursors to the ephedrine alkaloids. These metabolites are only present in young *E. sinica* stems and not in mature stems or roots. The related *Ephedra foemina* and *Ephedra foliata* also lack ephedrine alkaloids and their metabolic precursors in their aerial parts. A marked diversity in the ephedrine alkaloids content and stereochemical composition in 16 different *E. sinica* accessions growing under the same environmental conditions was revealed, indicating genetic control of these traits. The accessions can be classified into two groups according to the stereochemistry of the products accumulated: a group that displayed only 1*R* stereoisomers, and a group that displayed both 1*S* and 1*R* stereoisomers. (*S*)-cathinone reductase activities were detected in *E. sinica* stems capable of reducing (*S*)-cathinone to (1*R*,2*S*)-norephedrine and (1*S*,2*S*)-norpseudoephedrine in the presence of NADH. The proportion of the diastereoisomers formed varied according to the accession tested. A (1*R*,2*S*)-norephedrine *N*-methyltransferase capable of converting (1*R*,2*S*)-norephedrine to (1*R*,2*S*)-ephedrine in the presence of *S*-adenosylmethionine (SAM) was also detected in *E. sinica* stems. Our studies further support the notion that 1-phenylpropane-1,2-dione and (*S*)-cathinone are biosynthetic precursors of the ephedrine alkaloids in *E. sinica* stems and that the activity of (*S*)-cathinone reductases directs and determines the stereochemical branching of the pathway. Further methylations are likely due to *N*-methyltransferase activities.

1. Introduction

Ephedra sinica Stapf (Ephedraceae) is a traditional Chinese medicinal plant (Chinese name: *Ma Huang*) cultivated in China, Korea and Japan (Bruneton, 1995). Dried *E. sinica* stems are traditionally used as a tea mainly for the treatment of asthma, fever, coughs, lack of sweating, urination promotion and alleviating edema (Bensky et al., 1986; Bruneton, 1995). The main active principles of *E. sinica* are the unique and taxonomically restricted adrenergic agonists phenylpropylamino alkaloids, also known as the ephedrine alkaloids: (1*R*,2*S*)-norephedrine (1*S*,2*S*)-norpseudoephedrine, (1*R*,2*S*)-ephedrine, (1*S*,2*S*)-pseudoephedrine, (1*R*,2*S*)-*N*-methylephedrine and (1*S*,2*S*)-*N*-methylpseudoephedrine (Bruneton, 1995). In general, ephedrine alkaloids mimic the action of adrenaline both by direct agonist activity as well as by indirect release of norepinephrine via a carrier-mediated exchange mechanism resulting in the release of endogenous catecholamines from the post-ganglionic sympathetic fibers (Bruneton, 1995; Rothman et al., 2003). (1*R*,2*S*)-Ephedrine and (1*R*,2*S*)-norephedrine are also used in Western medicine for their vasoconstrictive properties, to treat acute asthma attacks as well as rhinitis, sinusitis and rhinopharyngitis (Bruneton, 1995). (1*R*,2*S*)-Ephedrine is also widely used during anesthesia, it is frequently prescribed for treatment of bronchial asthma, allergy and countering an overdose of depressants (Lewis and Elvin-Lewis, 1977). (1*S*,2*S*)-Pseudoephedrine is used to treat nasal congestion (Bruneton, 1995). It has been previously established that (1*R*,2*S*)-ephedrine is the major alkaloid accumulated by *E. sinica* while in other species such as *Ephedra*

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intermedia and *Ephedra lomatolepis* the predominant alkaloid is (1*S*,2*S*)-pseudoephedrine (Cui et al., 1991). *Ephedra foemina* and *Ephedra foliata* reportedly lack (1*R*,2*S*)-ephedrine and (1*S*,2*S*)-pseudoephedrine (Caveney et al., 2001). Although the pharmacological effects of ephedrine alkaloids have been well studied (Bruneton, 1995; Rothman et al., 2003), very little is known about the catalytic mechanisms by which plants synthesize such compounds.

Pulse chase radiolabeling experiments using *Ephedra gerardiana sikkimensis* stems allowed the preliminary elucidation of the ephedrine alkaloids metabolic pathway (Grue-Sorensen and Spenser, 1994). It was established that L-phenylalanine serves as the initial precursor for the ephedrine alkaloids biosynthesis (Grue-Sorensen and Spenser, 1994; Leete, 1958). These experiments suggested that ephedrine alkaloids are biosynthesized by condensation of pyruvate and benzoic acid to form the taxonomically restricted volatile compound 1-phenylpropane-1,2-dione (Fig. 1). 1-Phenylpropane-1,2-dione undergoes transamination to release the next committed intermediate of the pathway, namely (*S*)-cathinone, which is subsequently reduced to form (1*S*,2*S*)-norpseudoephedrine and (1*R*,2*S*)-norephedrine (Fig. 1). This pathway is similar to that postulated to occur in *Catha edulis*, a taxonomically distant plant that also accumulates ephedrine alkaloids (Grue-Sorensen and Spenser, 1994; Leete, 1958). In *Ephedra* spp. these initial steps were postulated to be followed by *N*-methylation reactions giving rise initially to (1*S*,2*S*)-pseudoephedrine and (1*R*,2*S*)-ephedrine, respectively, followed by a second *N*-methylation reaction producing (1*S*,2*S*)-*N*-methylpseudoephedrine and (1*R*,2*S*)-*N*-methylephedrine (Fig. 1) (Grue-Sorensen and Spenser, 1994). However, the only enzymatic reaction in this pathway that has been reported is the formation of *trans*-cinnamic acid from L-phenylalanine by the action of phenylalanine ammonia lyase (PAL) (Fig. 1). Also the first two intermediates, namely 1-phenylpropane-1,2-dione and (*S*)-cathinone had not been detected in naturally occurring *Ephedra* species, except for the detection of (*S*)-cathinone following feeding of *E. gerardiana sikkimensis* stems with labeled 1-phenylpropane-1,2-dione (Grue-Sorensen and Spenser, 1994).

In this study we report the detection of the first two intermediates of the proposed pathway to ephedrine alkaloids in young *E. sinica* stems, namely 1-phenylpropane-1,2-dione and (*S*)-cathinone (Fig. 1). We also report on inter- and intraspecies genetic polymorphism in ephedrine alkaloids composition and content in *Ephedra*. We present evidence for the existence of two novel enzymatic activities in *E. sinica* stems capable of reducing (*S*)-cathinone to (1*R*,2*S*)-norephedrine and (1*S*,2*S*)-norpseudoephedrine at different stereochemical ratios as well as a novel enzymatic activity yielding (1*R*,2*S*)-ephedrine by *N*-methylation of (1*R*,2*S*)-norephedrine in the presence of SAM.

2. Results

We employed a pathway-oriented targeted metabolomics approach as part of our strategy to enable the detection of the ephedrine alkaloids pathway components in *Ephedra*. Extracts of young freshly picked *E. sinica* stems were analyzed by GC–MS using our previously developed method for extraction of ephedrine alkaloids from *C. edulis* (Krizevski et al., 2008). This method has enabled the detection of the putative biosynthetic precursors 1-phenylpropane-1,2-dione and (*S*)-cathinone in young *E. sinica* stems for the first time as well as the ephedrine alkaloids and acetaldehyde conjugation products 2,4-dimethyl-5-phenyloxazolidine and 2,3,4-trimethyl-5-phenyloxazolidine (Fig. 2). None of the ephedrine alkaloids nor their conjugates and putative metabolic precursors were detected in *E. foemina* and *E. foliata* stems (Fig. 2). The compounds were identified by comparison of their MS and retention times to that of commercial and synthesized standards, extracted using the same procedure used for the extraction of compounds from plant material (Figs. 2 and S1). Four additional *E. sinica* accessions were analyzed for the presence of ephedrine alkaloids and their precursors (Table 1). Indeed, 1-phenylpropane-1,2-dione and (*S*)-cathinone were detected in young stems of all four accessions in varying quantities, with accession C accumulating the highest levels (0.7 ± 0.1 and 5 ± 0.8 μg/g fr. wt 1-phenylpropane-1,2-dione and (*S*)-cathinone, respectively (Table 1)). Young stems contained lower amounts (7-fold less) of the ephedrine alkaloids relative to mature stems (Table 1). Similar trends were observed for 2,4-dimethyl-5-phenyloxazolidine and 2,3,4-trimethyl-5-phenyloxazolidine as well (Table 1). None of the *E. sinica* accessions analyzed contained any of the ephedrine alkaloids or their putative metabolic precursors in root tissues (Table 1).

Considerable variation was detected in the ephedrine alkaloids content in 16 *E. sinica* accessions grown under the same environmental conditions concentrations ranging from 1.6 to 26 mg/g fr. wt (Fig. 3A). Interestingly, the different accessions can be grouped according to the stereochemistry of the products they accumulate. The first group accumulates almost exclusively (>96%) the (1*R*,2*S*) components norephedrine, ephedrine and *N*-methylephedrine out of the total ephedrine alkaloids content. The second group consists of accessions that accumulate both diastereoisomers in different ratios (Fig. 3A). Although the oxazolidines related to the ephedrine alkaloids were accumulated in lower levels as compared to their non-conjugated analogs, ranging from 0.1 to 0.9 mg/g fr. wt, similar trends were reflected in their stereochemistry (Fig. 3B). The accumulation patterns of ephedrine alkaloids and their oxazolidine conjugates observed strongly suggests the existence of different reductase activities capable of converting (*S*)-cathinone to (1*R*,2*S*)-norephedrine and (1*S*,2*S*)-norpseudoephedrine, respectively. Indeed, cell-free soluble protein extracts derived from *E. sinica* young stems were capable of catalyzing the reduction of (*S*)-cathinone using NADH as a coenzyme, giving rise to (1*R*,2*S*)-norephedrine and (1*S*,2*S*)-norpseudoephedrine (Fig. 4). Still, the accessions differed in the stereochemistry of the products formed. The enzymatic activity extracted from accession A produced mainly (1*R*,2*S*)-norephedrine (Fig. 4A), while the enzymatic activity extracted from accession B yielded both (1*S*,2*S*)-norpseudoephedrine and (1*R*,2*S*)-norephedrine at comparable levels (Fig. 4B). Heat inactivated proteins did not yield any of the biosynthetic products. NADPH could not be utilized as a coenzyme in these reactions (Fig. 4C).

The ephedrine alkaloids differ in the methylation patterns of their terminal amino group (Fig. 1). In norephedrine, the terminal amino group is not methylated while in ephedrine, one *N*-methylation is manifested. Methylephedrine is the result of two *N*-methylations displaying a tertiary terminal amino group. In parallel, the corresponding (1*S*,2*S*) diastereoisomers also display different degrees of methylation: norpseudoephedrine, displays no *N*-methylations at the terminal amino group, pseudoephedrine, displays one *N*-methylation and methylpseudoephedrine displays two *N*-methylations (see Fig. 1). *E. sinica* accessions also differ in the methylation patters for each of the (1*R*,2*S*) and (1*S*,2*S*) diastereoisomers (Fig. 5). Within the (1*R*,2*S*) stereoisomers, norephedrine is accumulated at much lower levels compared to its methylated derivatives ephedrine and *N*-methylephedrine in all accessions (Fig. 5). In parallel, the (1*S*,2*S*) stereoisomers methylation patterns were more complex. Three chemotypical groups can be distinguished as follows. Type I, that included accessions P, Q and C, that accumulated almost exclusively (1*S*,2*S*)-methylated derivatives (Fig. 5). Another group that we termed Type III included accessions N, H, F, K, G, and J, accumulated mainly (1*S*,2*S*)-non-methylated derivatives. An intermediate type that included accessions I, B, A, D, O, M, and R exhibited intermediate methylation patterns (20–

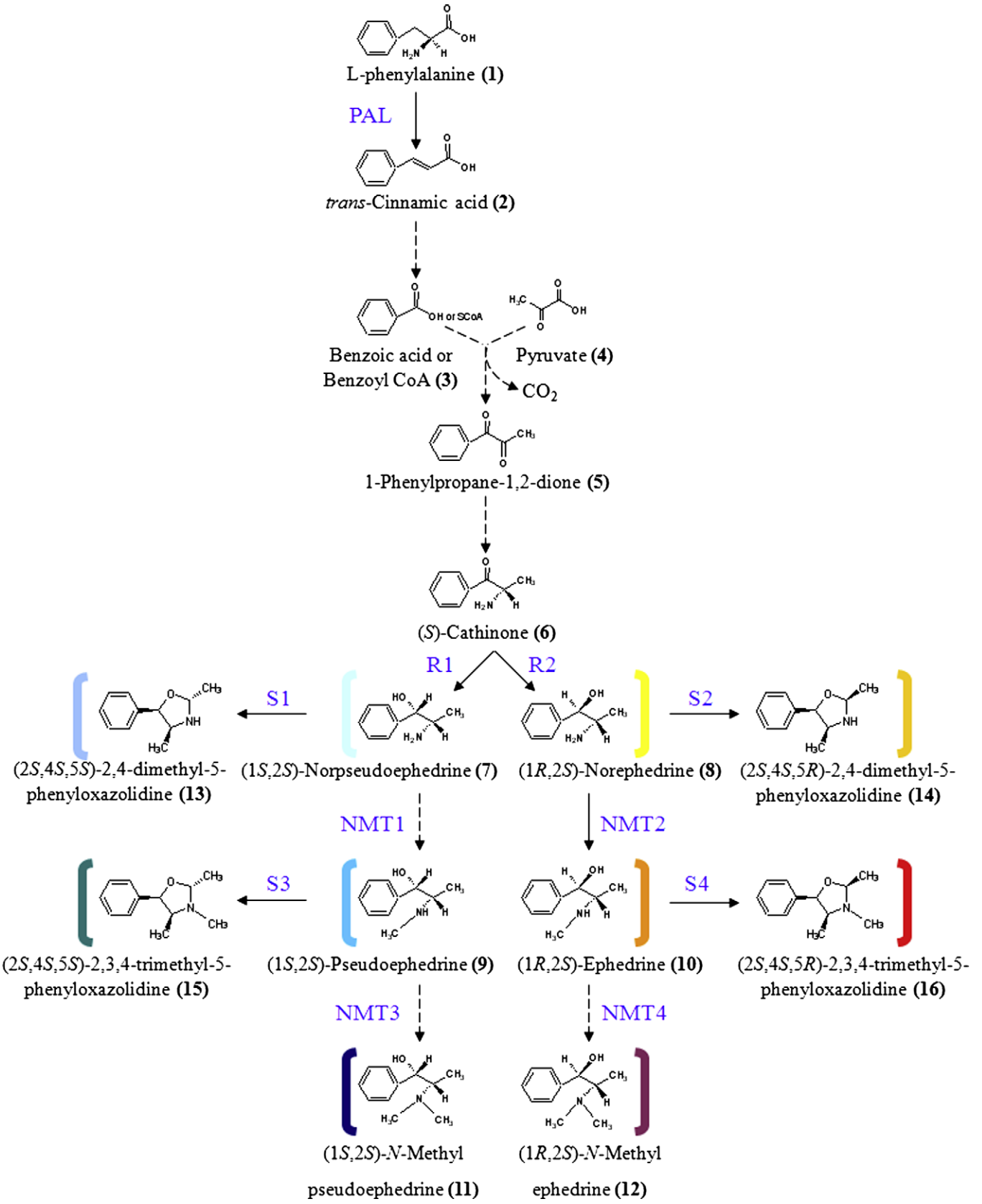

Fig. 1. The proposed biosynthetic pathway to ephedrine alkaloids in *E. sinica*. The pathway is based on early radiolabeling experiments (Grue-Sorensen and Spenser, 1994) and the presence of putative precursors (Fig. 2). Solid arrows represent biochemically resolved reactions including PAL (Okada et al., 2008) R1, R2 and NMT2 (Figs. 4 and 6) while dashed arrows represent biochemically unconfirmed reactions. The earlier steps from *trans*-cinnamic acid to benzoic acid, the formation of 1-phenylpropane-1,2-dione and the amine donor for the biosynthesis of (*S*)-cathinone are still unknown. **S1**–**4** represent spontaneous conjugations of acetaldehyde to ephedrine alkaloids.

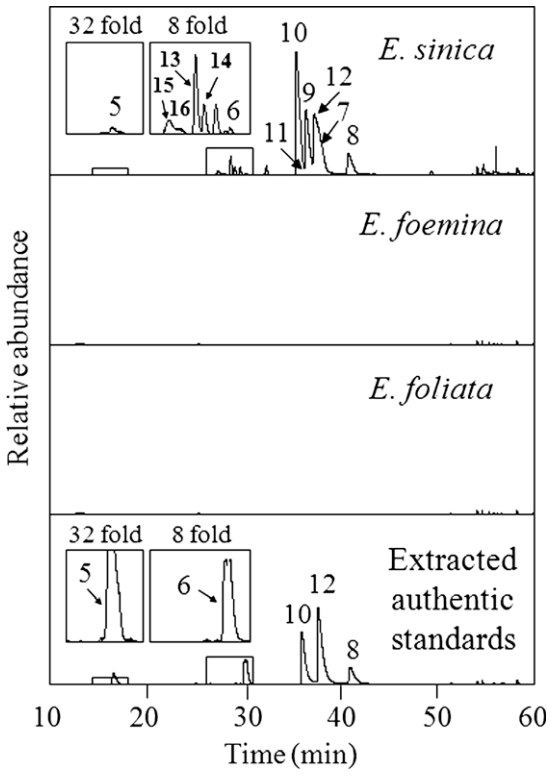

Fig. 2. GC–MS analysis of ephedrine alkaloids and their metabolic precursors 1-phenylpropane-1,2-dione and (*S*)-cathinone from young *E. sinica*, *E. foemina* and *E. foliata* stems in comparison to commercially available and synthetic standards extracted in a similar way to that of plant material. Numbers were assigned according to the metabolic pathway shown in Fig. 1. Compounds were identified by comparison of their retention times and mass spectra (Fig. S1) to that of commercially available and synthetic standards.

50% methylated (1*S*,2*S*) derivatives) was termed Type II (Fig. 5). The differential methylation patterns between the two branches of the ephedrine alkaloids biosynthetic pathway in the different accessions implies that a similar but not identical enzymatic methylation mechanism could be involved in each stereoisomeric branch of the pathway (Fig. 5).

A methyltransferase activity was successfully extracted from young *E. sinica* stems. This activity catalyzed the conversion of (1*R*,2*S*)-norephedrine to (1*R*,2*S*)-ephedrine in the presence of SAM as the methyl donor (Fig. 6A). The product (1*R*,2*S*)-ephedrine was not detected when a heat inactivated protein extract was used (Fig. 6B), nor in the absence of SAM (Fig. 6C).

3. Discussion

Many studies addressing the chemical composition of *E. sinica* products have been reported (Cui et al., 1991; Hou et al., 2007; Jiang et al., 2007; Kitani et al., 2009; Okada et al., 2009). In most cases only processed dietary supplements or mature dried *E. sinica* stems (the parts traditionally used and not young fresh stems) have been analyzed for their ephedrine alkaloids composition and content (Cui et al., 1991; Hou et al., 2007; Jiang et al., 2007; Kitani et al., 2009; Okada et al., 2009). Thus, these analyses have only contributed to our understanding of the biochemical pathway in a very limited way. We have analyzed fresh young *E. sinica* tissues and found 1-phenylpropane-1,2-dione and (*S*)-cathinone (Fig. 2, Table 1), metabolites that have been implicated as biochemical precursors of the ephedrine alkaloids but had not been previously found in the materials routinely analyzed. These initial precursors are also found in young tissues of *C. edulis* (Celastraceae) (Krizevski et al., 2007, 2008) a taxonomically unrelated plant that also accumulates ephedrine alkaloids, apparently using a similar biosynthetic pathway (Grue-Sorensen and Spenser, 1994; Leete, 1958). Similarly to what we found in *E. sinica* tissues (Table 1), the first two putative precursors namely 1-phenylpropane-1,2-dione and (*S*)-cathinone were detected only in fresh young *C. edulis* tissues (Krizevski et al., 2007, 2008). These intermediates are apparently further metabolized in *C. edulis* upon tissue maturation in a parallel way to that reported here for *E. sinica* (Krizevski et al., 2007, 2008). Although fresh young *E. sinica* stems contain significant levels 1-phenylpropane-1,2-dione and (*S*)-cathinone, both of these compounds are absent in mature stems (Fig. 2 and Table 1). Moreover, mature stems accumulated higher levels (7-fold) of ephedrine alkaloids relative to young stems, suggesting that biosynthesis occurs during stem maturation, but with a rapid conversion of pathway intermediates to end products (Table 1). This is also supported by observations indicating low ephedrine concentrations in apical meristems, and increased levels in lower parts of the stems (O'Dowd et al., 1998). Previous studies have indicated that *E. foemina* and *E. foliata* lack (1*S*,2*S*)-pseudoephedrine and (1*R*,2*S*)-ephedrine (Caveney et al., 2001). We have demonstrated that all of the ephedrine alkaloids, their oxazolidine derivatives and metabolic precursors are absent in these species (Fig. 2), fur-

Table 1
Levels of ephedrine alkaloids and 1-phenylpropane-1,2-dione in fresh *E. sinica* tissues. The data (μg/g fr. wt) presented are an average of five replicates ±s.e. Compounds that were not detected are marked N.D. Asterisks denote the oxazolidine ring numbering system.

Compound	Accession A		Accession B		Accession C		Accession D	
	Young stems	Mature stems	Young stems	Mature stems	Young stems	Mature stems	Young stems	Mature stems
Precursors								
1-Phenylpropane-1,2-dione	0.2 ± 0.04	N.D.	0.4 ± 0.03	N.D.	0.7 ± 0.1	N.D.	0.1 ± 0.02	N.D.
(*S*)-cathinone	1 ± 0.2	N.D.	4 ± 0.4	N.D.	5 ± 0.8	N.D.	1 ± 0.3	N.D.
(1R)-compounds								
(1*R*,2*S*)-norephedrine	95 ± 18	116 ± 27	155 ± 16	408 ± 105	145 ± 10	401 ± 127	148 ± 43	722 ± 311
(2*S*,4*S*,5*R*)-2,4-dimethyl-5-phenyloxazolidine*	8 ± 1.4	98 ± 29	11 ± 1	80 ± 26	8 ± 0.8	41 ± 10	8 ± 1.6	91 ± 42
(1*R*,2*S*)-ephedrine	201 ± 33	1117 ± 155	516 ± 98	4120 ± 183	232 ± 34	3551 ± 289	191 ± 53	2190 ± 698
(2*S*,4*S*,5*R*)-2,3,4-trimethyl-5-phenyloxazolidine*	2.4 ± 0.4	23 ± 6	2 ± 0.1	11 ± 1	N.D.	N.D.	1.7 ± 0.3	18 ± 5
(1*R*,2*S*)-*N*-methylephedrine	5 ± 0.6	175 ± 31	10 ± 3	355 ± 16	8 ± 2	582 ± 54	3 ± 0.8	113 ± 35
(1S)-compounds								
(1*S*,2*S*)-norpseudoephedrine	11 ± 3	31 ± 4	428 ± 90	1923 ± 181	712 ± 36	2782 ± 339	80 ± 19	467 ± 207
(2*S*,4*S*,5*S*)-2,4-dimethyl-5-phenyloxazolidine*	1.5 ± 0.2	24 ± 6	15 ± 3.5	254 ± 75	27 ± 4	625 ± 199	9 ± 1.7	133 ± 64
(1*S*,2*S*)-pseudoephedrine	3 ± 0.6	23 ± 3	110 ± 33	1671 ± 149	370 ± 26	6344 ± 545	30 ± 8	347 ± 93
(2*S*,4*S*,5*S*)-2,3,4-trimethyl-5-phenyloxazolidine*	0.4 ± 0.04	6 ± 1	3.7 ± 0.8	57 ± 9	7 ± 1	187 ± 32	1.4 ± 29	25 ± 9
(1*S*,2*S*)-*N*-methylpseudoephedrine	N.D.	2 ± 0.7	0.6 ± 0.17	13 ± 1.5	0.9 ± 0.16	62 ± 8.7	0.25 ± 0.1	5 ± 2.3

None of the compounds listed in the table were detected in roots of any of the accessions analyzed.

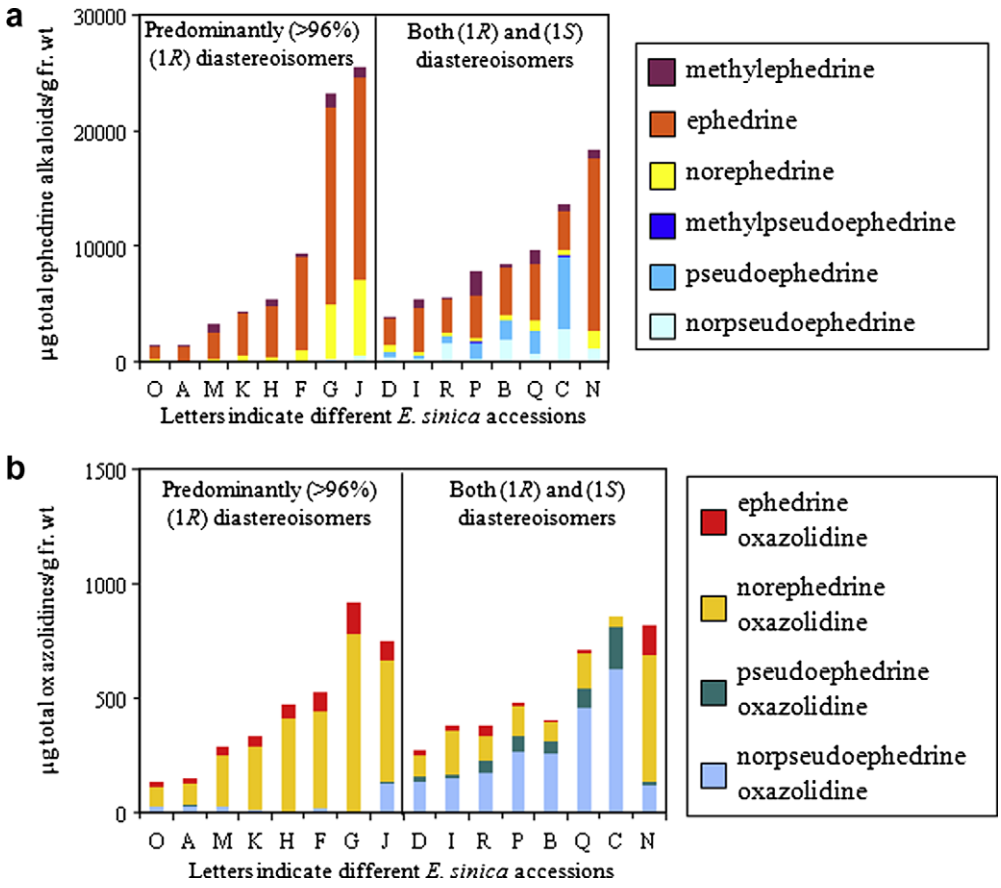

Fig. 3. Levels of ephedrine alkaloids in fresh *E. sinica* mature stems. A: Ephedrine alkaloids. B: Oxazolidines. The letters represent the accessions analyzed. Colors are according to Fig. 1. The data presented (μg/g fr. wt) are an average of five replicates ±s.e.

ther strengthening the possibility that the proposed pathway (Fig. 1) is operational.

It has been suggested that the initial conversion of L-phenylalanine into the ephedrine alkaloids takes place in roots (Okada et al., 2008). This hypothesis is based on the isolation of four isoforms of *E. sinica* phenylalanine ammonia lyase (PAL), a key enzyme in the phenylpropanoid pathway to lignin and flavonoids that has also been implicated in the ephedrine alkaloids biosynthetic pathway (Okada et al., 2008) (Fig. 1). Interestingly, roots lack both ephedrine alkaloids and their metabolic precursors (Table 1). The presence of 1-phenylpropane-1,2-dione and (*S*)-cathinone in young shoots (but not in roots) suggests that the later steps in ephedrine alkaloid biosynthesis are likely to occur in stems. A similar situation is apparent in *C. edulis*, where roots also lack the putative precursors (Krizevski et al., 2007). This is different to the biosynthesis of pyridine and tropane alkaloids that takes place in Solanaceae roots, where nicotine and hyoscyamine are subsequently transported to leaves (Drager, 2007).

Phenylethylamino alkaloids (such as tyramine and dopamine) could be regarded as a structurally similar group of alkaloids to the ephedrine alkaloids. However, the biosynthesis of phenylethylamino alkaloids occurs via simple decarboxylation of aromatic amino acids (Kaminaga et al., 2006) while that of the ephedrine alkaloids is much more complex (Fig. 1). It has been previously established that ephedrine alkaloids can undergo spontaneous conjugation with different aldehydes *in vitro* resulting in the formation of pharmacologically active oxazolidines (Beckett and Jones, 1977; Walker et al., 1996, 1998; Johansen and Bundgaard, 1982; Moloney et al., 1997; Khruscheva et al., 1997). The *in vivo* occurrence of such conjugates in *Ephedra* has not been verified. We have detected (2*S*,4*S*,5*S*)-2,4-dimethyl-5-phenyloxazolidine, (2*S*,4*S*,5*R*)-2,4-dimethyl-5-phenyloxazolidine, (2*S*,4*S*,5*S*)-2,3,4-trimethyl-5-phenyloxazolidine and (2*S*,4*S*,5*R*)-2,3,4-trimethyl-5-phenyloxazolidine as conjugation products of ephedrine alkaloids and acetaldehyde in *E. sinica* extracts (Fig. 2). These oxazolidine derivatives were absent in samples of authentic standards, that underwent a similar extraction and analyses procedures as compared to plant samples. Thus, although the conjugation might be non-enzymatic, the possibility of acetaldehyde contamination during the sample preparation procedures is unlikely and indicates that the source of the acetaldehyde is probably the plant tissues (Fig. 2). Acetaldehyde is known to be a major plant metabolite produced and emitted during night time and produced during re-aeration of roots after their submergence (Karl et al., 2002; Tsuji et al., 2003).

Only a few reports addressing natural polymorphism in the ephedrine alkaloid composition and content in *E. sinica* are known. These analyses were performed on plants collected from wild populations (Cui et al., 1991; Kitani et al., 2009; Wang et al., 2010). Therefore, the relative contribution of environmental versus genetic factors affecting the ephedrine alkaloids profiles in *Ephedra* has not been fully evaluated. Our profiling of 16 different *E. sinica* accessions growing under similar environmental conditions (Figs. 3 and 5) indicated a high variability in the ephedrine alkaloids composition and content between the different accessions. Our data suggest therefore that genetic factors are involved in determining the ephedrine alkaloids profiles. It has been noted before that *E. sinica* plants growing in areas with low humidity

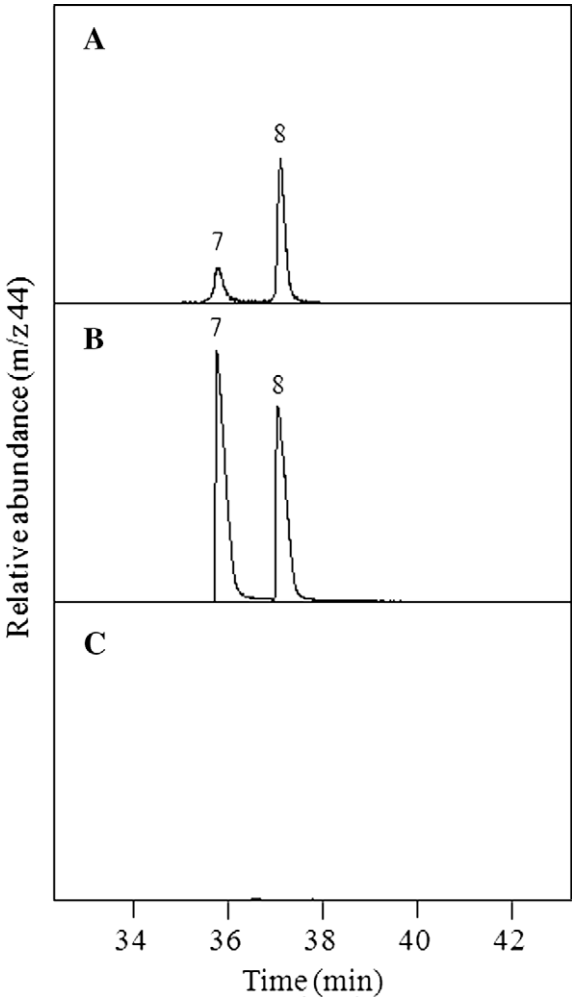

Fig. 4. Cathinone reductase activity in young *E. sinica* stems. Cell-free protein extracts were incubated with (*S*)-cathinone and NADH. A: Complete assay of extracts derived from accession A. B: Complete assay of extracts derived from accession B. C: Heat inactivated enzyme derived from accession A (other controls such as heat inactivated protein extracts from accession B, as well as complete assays lacking (*S*)-cathinone or NADH, as well as incubations with NADPH instead of NADH were devoid of activity (not shown). The enzymatic reaction was analyzed using GC–MS and the products (1*S*,2*S*)-norpseudoephedrine **(7)** and (1*R*,2*S*)-norephedrine **(8)** were identified by comparison of their retention times and mass spectra to that of authentic (1*R*,2*S*)-norephedrine standard. The experiment was repeated twice with similar results.

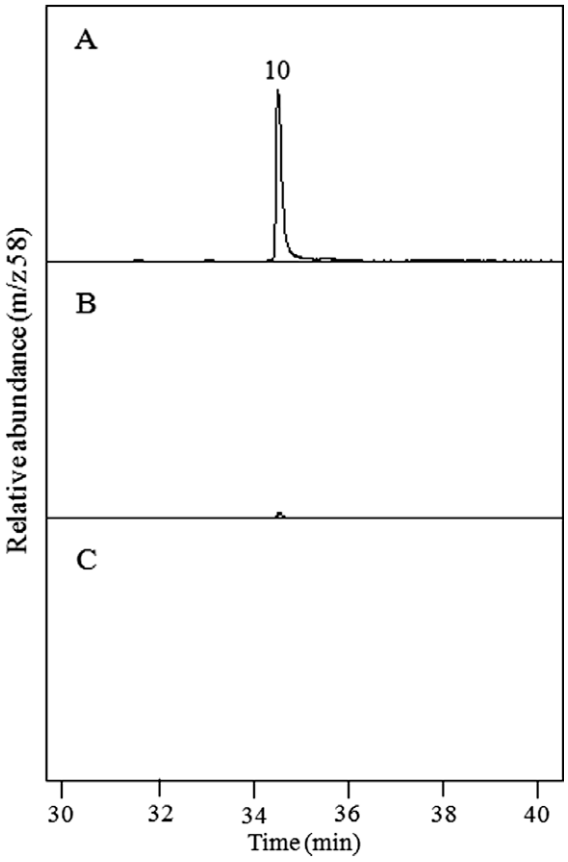

Fig. 6. Norephedrine *N*-methyltransferase activity in young *E. sinica* stems. Cell-free protein extracts were incubated with norephedrine and SAM. A: Complete assay. B: Heat inactivated enzyme. C: Complete assay in the absence of SAM. The enzymatic reaction was analyzed by GC–MS and the product (1*R*,2*S*)-ephedrine **(10)** was identified by comparison of its retention time and mass spectrum to that of authentic standard. The experiment was repeated twice with similar results.

display lower ephedrine alkaloids levels as compared to plants growing in high humidity conditions (Yaniv and Palevitch, 1982; O'Dowd et al., 1998). Moreover, apical tips accumulate lower levels of ephedrine alkaloids than basal woodier tissues, whereas internodes contain higher alkaloid levels than nodes (O'Dowd et al., 1998). Most *Ephedra* species accumulate ephedrine alkaloids albeit in different levels with different enantiomeric ratios within each pair of diastereoisomers (Cui et al., 1991; Caveney et al., 2001). Moreover, the ephedrine alkaloids profiles of *E. sinica* plants growing in different regions of Mongolia and China included high levels of the (1*R*,2*S*)-diastereoisomers (norephedrine, ephedrine and *N*-methylephedrine) reaching up to 97% of the total ephedrine alkaloids and low levels of (1*R*,2*S*)-compo-

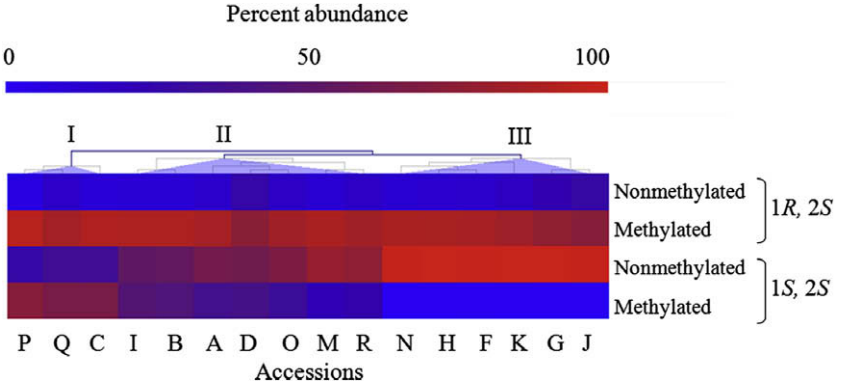

Fig. 5. Hierarchical clustering analysis of ephedrine alkaloids methylation patterns in *E. sinica*.

nents of about 12% (Kitani et al., 2009; Wang et al., 2010). Since our sampling populations were grown under identical climatic conditions, our data indicates that these variations are directed by genetic factors. Our alkaloid profiling has also indicated the presence of two *E. sinica* chemotypes. The "optically pure" chemotype (represented by accessions A, F, G, H, J, K, M and O) accumulated very high levels of (1*R*,2*S*) alkaloids (Fig. 3). The second "mixed" chemotype (represented by accessions B, C, D, I, N, P, Q and R) accumulated both (1*R*,2*S*) and (1*S*,2*S*) alkaloids (Fig. 3). Interestingly (*S*)-cathinone reductase activities that operate at the branching point of the pathway (Fig. 1 reactions R1 and R2) differed in the two accessions analyzed. The (*S*)-cathinone reductase activity extracted from accession A (representing the optically pure chemotypes), was able to produce mainly (1*R*,2*S*)-norephedrine from (*S*)-cathinone and NADH (Fig. 4A). While the (*S*)-cathinone reductase activity extracted from accession B (representing the "mixed" chemotype), catalyzed the formation of both diastereoisomers (1*R*,2*S*)-norephedrine and (1*S*,2*S*)-norpseudoephedrine at equivalent levels (Fig. 4B). Interestingly, comparable activities are present in young *C. edulis* leaves capable of reducing (*S*)-cathinone (utilizing both NADPH and NADH with the later displaying a higher efficiency) to form (1*S*,2*S*)-norpseudoephedrine and (1*R*,2*S*)-norephedrine (Fig. S2; Krizevski et al., 2007). Stereoselective reduction mechanisms are known in the plant kingdom. They include oxidoreductase enzymes in peppermint (*Mentha* × *piperita*, Lamiaceae), catalyzing the reduction of (2*S*,5*R*)-menthone either to (1*R*,2*S*,5*R*)-menthol by (2*S*,5*R*)-menthone reductase 1 or to its stereoisomer (1*S*,2*S*,5*R*)-neomenthol by (2*S*,5*R*)-menthone reductase 2 (Davis et al., 2005). In opium poppy (*Papaver somniferum*, Papaveraceae) a reductase capable of reducing salutaridine to (7*S*)-salutaridinol but not to its stereoisomer (7*R*)-*epi*-salutaridinol has been described (Ziegler et al., 2006). In *Hyoscyamus niger* (Solanaceae) tropinone can also be stereospecifically reduced by two distinct tropinone reductases to either (*S*)-tropine or to (*R*)-pseudotropine (Nakajima et al., 1993). Interestingly, a bacterial short chain alcohol dehydrogenase reductase reduced *N*-methylated (*S*)-cathinone to (1*S*,2*S*)-pseudoephedrine (but not to (1*R*,2*S*)-ephedrine) in the presence of NADPH (Kataoka et al., 2006, 2008). In light of our and other data we propose that *Ephedra* stems contain at least two reductases that differ in the stereoselective conversion of (*S*)-cathinone to either (1*S*,2*S*)-norpseudoephedrine or (1*R*,2*S*)-norephedrine (Fig. 1).

We have provided evidence that an *N*-methyltransferase activity extracted from young *E. sinica* stems converts (1*R*,2*S*)-norephedrine to (1*R*,2*S*)-ephedrine in the presence of SAM (Fig. 6). The differential methylation patterns as related to the stereochemistry of each branch of the ephedrine alkaloids metabolic pathway (Fig. 5) might imply that additional stereoselectivity is involved in the methylation steps. Our data indicates that methylation of the (1*R*,2*S*) stereoisomers is common in all accessions, but in general, methylation of the (1*S*,2*S*) stereoisomers is related to their availability as substrates as is apparently dictated by the reductase enzymes. More experimental work is needed to uncover actual substrate availabilities and additional NMT activities from *Ephedra* and comprehensively determine their substrate specificities. Methyltransferases generally accept various substrates (Lewinsohn et al., 2000; Choi et al., 2001, 2002; Gross et al., 2002; Scalliet et al., 2008). In some cases the reactions are stereospecific, such as the *Tinospora cordifolia* (Menispermaceae) (*S*)-coclaurine *N*-methyltransferase, a stereoselective enzyme methylating (*S*)-norcoclaurine and (*S*)-coclaurine but not (*R*)-norcoclaurine and (*R*)-coclaurine (Loeffler et al., 1994). Conversely, the coclaurine *N*-methyltransferase isolated from *Coptis japonica* (Ranunculaceae) utilizes both (*S*)-norcoclaurine and (*R*)-norcoclaurine as well as (*S*)-coclaurine and (*R*)-coclaurine as substrates (Choi et al., 2001, 2002).

4. Concluding remarks

Metabolic precursors of ephedrine alkaloids have been detected in young *E. sinica* stems and the involvement of genetic factors in ephedrine alkaloids accumulation has been established. Additionally, we have successfully extracted (*S*)-cathinone reductase activities that might determine chemotypic differentiation and a (1*R*,2*S*)-norephedrine *N*-methyltransferase that converts (1*R*,2*S*)-norephedrine into (1*R*,2*S*)-ephedrine. This work is part of our ongoing attempts to establish a chemical, biochemical and genomic platform, as well as a unique germplasm collection to facilitate the study of the ephedrine alkaloids biosynthesis in *E. sinica*. These advances will contribute to our understanding of this little studied biosynthetic pathway, and will eventually enable the production of plants with desirable alkaloids profiles for herbal medicine users as well as for the pharmaceutical industry.

5. Experimental

5.1. Plant material and chemical standards

E. foemina plant material was collected from a wild population growing in Kibbutz Yifat, Jezreel valley Northern Israel and *E. foliata* plant material was collected from a wild population growing 10 km north of the city of Eilat in Southern Israel. *E. sinica* seeds were purchased from Horizon Herbs, Williams, OR, USA. The seeds were originally collected from wild open pollinated *E. sinica* populations in northern China. Seeds were germinated and planted in 20 l pots containing a compost and tuff mixture. *Ephedra* plants were grown outdoors at the Newe Ya'ar Research Center in Northern Israel under commercial growing practices which include drip irrigation and fertilization. The plants used in this study were 3 years old. The variation along mature stems was previously established: apical tips accumulate lower levels of ephedrine alkaloids than basal woodier tissues and internodes contain higher alkaloid levels than nodes (O'Dowd et al., 1998). Thus, strict and even sampling procedure was used in order to accurately analyze ephedrine alkaloids from *Ephedra* plant material based upon analyzing entire stems with uniform age and size. Light green stems freshly emerging during the spring were considered "young" and dark green stems that emerged in the previous spring were considered "mature". Authentic standards of 1-phenyl-1,2-propanedione, (1*R*,2*S*)-norephedrine, (1*R*,2*S*)-ephedrine, (1*R*,2*S*)-*N*-methylephedrine and the internal standard caffeine were purchased from Sigma Chem. Co., St Louis, MO USA, acetaldehyde was purchased from Fluka AG Buchs, Switzerland, while (*S*)-cathinone was synthesized by oxidation of (1*R*,2*S*)-norephedrine using $KMnO_4$ (Krizevski et al., 2007). (2*S*,4*S*,5*R*)-2,4-dimethyl-5-phenyloxazolidine was synthesized from (1*R*,2*S*)-norephedrine and (2*S*,4*S*,5*R*)-2,3,4-trimethyl-5-phenyloxazolidine was synthesized from (1*R*,2*S*)-ephedrine (see below).

5.2. Chemical synthesis of (2S,4S,5R)-2,4-dimethyl-5-phenyloxazolidine and (2S,4S,5R)-2,3,4-trimethyl-5-phenyloxazolidine

(1*R*,2*S*)-norephedrine and acetaldehyde in a 1:20 M ratio were dissolved in 5 ml of 10% (w/v) Gum Arabic and the sample was vigorously shaken for 30 s. An equivalent volume of 5 ml 1 N NaOH was added to the sample together with 20 ml MTBE (methyl *tert*-butyl ether). The sample was then vigorously shaken for 30 s and centrifuged at 10*g* for 20 min in order to break the emulsion, resulting in a clear fraction separation. The top organic fraction was collected and the aqueous residue was reextracted with 20 additional milliliters of MTBE. The pooled ethereal extracts were

dried by the addition of anhydrous sodium sulfate and then evaporated under a gentle stream of nitrogen. The same procedure was repeated but using (1*R*,2*S*)-ephedrine and acetaldehyde. Structures were previously elucidated (Beckett and Jones, 1977).

5.3. Extraction of ephedrine alkaloids and metabolic precursors from fresh plant material

Freshly picked *Ephedra* stems (~0.5 g) or roots (as indicated), were ground with a mortar and pestle in the presence of liquid nitrogen. Three milliliters of H_2O containing 200 μg caffeine as internal standard (in parallel extractions, 200 μg (1*R*,2*S*)-ephedrine was used as external standard for quantification) were added to the fine powder, the sample was shaken for 30 min at 250 RPM and filtered through one layer of Miracloth (Calbiochem). Aliquot 1.5 ml of the sample was transferred into an 8 ml glass vial into which 1.5 ml of 1 N NaOH was added in order to retrieve the alkaloids in their uncharged form. Then, 3 ml of MTBE were added to the sample that was then vigorously shaken for 30 s and centrifuged at 10*g* for 20 min in order to break the emulsion, resulting in a clear fraction separation. The top organic fraction containing the alkaloids was collected and the aqueous residue was reextracted with 3 additional milliliters of MTBE. The pooled ethereal extracts were dried by the addition of anhydrous Na_2SO_4 and then evaporated under a gentle stream of N_2 to a final volume of 0.25 ml. One microliter was injected into the GC–MS instrument for analysis.

5.4. Gas chromatography–mass spectrometry of plant material

The GC–MS analysis of ephedrine alkaloids and their putative precursors was preformed with a Hewlett–Packard GCD gas chromatograph equipped with a WCOT Restek Rt-βDEXsm fused silica column (30 m × 0.25 mm × 0.25 μm). The gas chromatograph was operated in splitless injector mode using 6 min delay time. Helium was used as the carrier gas with a flow rate of 0.8 ml/min. The injector and detector temperatures were 230 °C. The oven was set to the following temperature program: 100 °C was the initial temperature held for 1 min; 1.5 °C/min up to 140 °C followed by 0 °C/min for 20 min and then 10 °C/min up to 220 °C held for 10 min. Compounds were identified by comparison of their retention times to extracted commercial and synthetic standards using baseline runs between samples (Krizevski et al., 2008).

5.5. Enzyme extractions

Both the cathinone reductases and norephedrine *N*-methyltransferase enzymes were extracted as follows. Young freshly picked *E. sinica* stems (20 g) were ground with a mortar and pestle under liquid nitrogen, with 2 g of analytical grade sea sand and 2 g polyvinylpolypyrrolidone (PVPP). The crushed powder was transferred to a 250 ml Erlenmeyer flask that was chilled on ice. Two hundred milliliters of 50 mM Bis–Tris propane buffer, pH 8.5 containing 10% (v/v) glycerol, 1% (w/v) polyvinylpyrrolidone (PVP-40) and 5 mM dithiothreitol were added. The sample was then filtered through one layer of Miracloth (Calbiochem) and centrifuged at 15,000*g* for 20 min at 4 °C. The resulting crude soluble protein fraction was transferred to a clean ice-chilled Erlenmeyer flask and a final 30% saturation ammonium sulfate was added to the sample followed by 1 h incubation at 4 °C. The sample was centrifuged at 15,000*g* for 20 min at 4 °C. The precipitated proteins and polyphenols were discarded and additional ammonium sulfate was added to reach a 60% saturation level followed by 1 h incubation at 4 °C. The sample was centrifuged at 15,000*g* for 20 min at 4 °C. The precipitated crude protein fraction was dissolved in 5 ml buffer. The remaining soluble fraction was transferred to a new Erlenmeyer flask and ammonium sulfate was again added reaching a 90% ammonium sulfate saturation level followed by 1 h incubation at 4 °C. The sample was centrifuged at 15,000*g* for 20 min at 4 °C. The precipitated crude protein fraction was dissolved in 5 ml buffer. Each precipitated protein fraction was dissolved in 5 ml buffer and desalted by gel permeation chromatography on a BioGel P-6 column at a flow rate of 1 ml/min using the same extraction buffer. Fractions of 1 ml volume were evaluated for their protein content using the Bradford reagent (Bradford, 1976), with bovine serum albumin (BSA) as standard. The first five fractions showing the highest levels of protein were combined and used in further experiments. Heat inactivated enzyme was obtained by boiling the enzyme in 100 °C for 20 min.

5.6. Cathinone reductase enzymatic assays

The enzyme assay reaction mixture for the reduction of (*S*)-cathinone consisted of 50 mM Bis–Tris propane buffer, pH 8.5 containing 10% (v/v) glycerol, 1% (w/v) polyvinylpyrrolidone (PVP-40), 5 mM dithiothreitol, 5 mM (*S*)-cathinone, 5 mM NADH and up to 0.5 ml of protein extract (approximately 3.5 mg protein, 30–60% ammonium sulfate fraction) in a total volume of 1 ml. After 1 h incubation at 37 °C the reaction was stopped by the addition of 1 ml 1 N NaOH. The alkaloids were extracted twice with 4 ml MTBE. The ether sample was then evaporated and treated as above and analyzed by GC–MS.

5.7. Norephedrine N-methyltransferase enzymatic assays

The enzyme assay reaction mixture for the methylation of (1*R*,2*S*)-norephedrine consisted of 50 mM Bis–Tris propane pH 8.5 containing 10% (v/v) glycerol, 1% (w/v) polyvinylpyrrolidone (PVP-40), 5 mM dithiothreitol, 5 mM (1*R*,2*S*)-norephedrine, 5 mM SAM and up to 0.5 ml protein extract (approximately 2 mg protein, 60–90% ammonium sulfate fraction) in a total volume of 1 ml. After an over-night incubation at 37 °C the reaction was stopped by the addition of 1 ml 1 N NaOH. The alkaloids were extracted twice with 4 ml MTBE. The ether sample was then evaporated and treated as above and analyzed by GC–MS.

5.8. Gas chromatography–mass spectrometry of enzymatic products

The analysis was preformed as above but using 100 °C as the initial temperature, held for 1 min; 1.5 °C/min up to 165 °C and then 10 °C/min up to 220 °C held for 10 min. Compounds were identified by comparison of their retention times to extracted authentic standards (Krizevski et al., 2008).

5.9. Hierarchical clustering

The analysis was performed on the data sets obtained from alkaloid profiling with the software package TMEV (Saeed et al., 2003) using the hierarchical clustering with Pearson correlation metric. Prior to the analysis data were calculated as the percentage of nonmethylated and methylated (both one and two methylations) compounds for each group of stereoisomers independently.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.03.019.

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