Heliyon

Received:
30 July 2017
Revised:
13 November 2017
Accepted:
23 November 2017

Cite as: Nahid Moghbel, BoMi Ryu, Angela Ratsch, Kathryn J. Steadman. Nicotine alkaloid levels, and nicotine to nornicotine conversion, in Australian *Nicotiana* species used as chewing tobacco. Heliyon 3 (2017) e00469.
doi: 10.1016/j.heliyon.2017.e00469

Nicotine alkaloid levels, and nicotine to nornicotine conversion, in Australian *Nicotiana* species used as chewing tobacco

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Abstract

A range of endemic *Nicotiana* species are chewed as a smokeless tobacco by several Aboriginal populations of Australia. In tobacco research, nicotine to nornicotine conversion is important because nornicotine lowers tobacco quality and is detrimental to health. A diverse group of cytochrome P450 genes with different transcriptional regulations are involved in this conversion. The primary aims of this study were to quantify the pyridine alkaloids and investigate nicotine to nornicotine conversion in laboratory-grown Australian *Nicotiana* spp. Nicotine, nornicotine, anatabine, anabasine, myosmine and cotinine were quantified in fresh leaves of 24 out of the 26 recognised Australian *Nicotiana* taxa. Conserved regions of CYP82E related genes were PCR amplified in all studied taxa. The conversion process in fresh leaves was compared with that in leaves that underwent a simulated curing process for species that we identified as being high converters (*N. cavicola, N. goodspeedii*, *N. velutina*) and low converters (*N. benthamiana, N. excelsior, N. gossei*). Agarose gel electrophoretic analysis of CYP82E related genes obtained from the PCR amplification of the cDNA in fresh versus leaves with simulated curing showed about a 3-fold increase in transcript accumulation levels in cured leaves of the high converter species, while the transcript

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

accumulation in *N. gossei* and *N. excelsior* maintained a steady basal level and increased by a small amount in *N. benthamiana*. This suggests the presence of functional loci that are triggered by curing in only high converter species and indicates a potential risk for chewers of high converter species.

Keywords: Natural product chemistry, Pharmaceutical science, Pharmaceutical chemistry, Toxicology, Plant biology, Biochemistry, Genetics

1. Introduction

All species of the genus *Nicotiana* L. have the characteristic feature of producing pyridine alkaloids, but the number and abundance of the different alkaloids are highly variable within the genus [1, 2]. While nicotine is considered to be the most abundant alkaloid (~95%) in *Nicotiana* species, there are many other structurally related pyridine alkaloids in various *Nicotiana* plants and their dried/cured leaves, primarily nornicotine, anabasine and anatabine. Nornicotine, the major alkaloid after nicotine in most species, is a demethylated derivative of nicotine. N'-demethylation of nicotine to nornicotine mainly occurs in the senescing leaves of *Nicotiana* plants. *Nicotiana* species that convert a large portion of their nicotine content to nornicotine during senescence and curing are described as “converters”, whereas the species that mainly accumulate nicotine in their leaves are “nonconverters” [3, 4].

In tobacco research, nicotine to nornicotine conversion has vital importance. This is because nornicotine affects tobacco quality by causing unwanted flavour and decreasing smoking quality [5]. Similar to nicotine, nornicotine accumulates in the brain, induces dopamine release and contributes to the pharmacological profile of nicotine [6]. However, nornicotine is believed to be a source of many undesirable health effects. The most important health implications attributed to nornicotine are due to it being the main precursor of N’-nitrosonornicotine (NNN), which is a tobacco specific nitrosamine (TSNA) with high potential in inducing cancer in laboratory animals [7, 8, 9]. Other than that, nornicotine induces the aberrant glycation of proteins within the cells and alters the pharmacological properties of the commonly prescribed steroid medication, prednisone [10]. It also catalyses retinal isomerization that is responsible for age-related macular degeneration, birth defects associated with smoking, and other smoking-associated abnormalities that stem from disruption of retinoid metabolism [11]. Nornicotine is also believed to be responsible for periodontal disease associated with smoking [12]. Research efforts have focussed on understanding the conversion phenomenon, and in breeding low nornicotine-producing species [13]. Nornicotine constitutes about 3–5% of the total alkaloid content in major commercial varieties of *Nicotiana* (mainly *N. tabacum*), but this could be up to 97% in high converter wild species or varieties of commercial tobacco [14, 15].

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

The molecular identity of the nicotine to nornicotine conversion factor in *Nicotiana* species has been of great importance, since the encoding of nicotine demethylase enzymes is the major determinant in formation and accumulation of nornicotine in these species. A group of cytochrome P450 genes, belonging to the 82E subfamily have been reported to be involved in encoding of these functional N-demethylases in *Nicotiana* spp. [4]. This group of P450 genes are differentially regulated between high converter and low or nonconverter *Nicotiana* [4]. In human liver microsomes, nicotine N-demethylation is catalysed by the P450 subfamilies CYP2A and CYP2B [16]. There are at least five CYP82E related genes in *N. tabacum*: CYP82E4, CYP82E5 and CYP82E10 encode functional nicotine N-demethylases [3, 4, 17] whereas CYP82E2 and CYP82E3 encode inactive enzymes [3]. Investigations involving conversion locus analysis in *Nicotiana* spp. are limited to the varieties of important commercial tobacco, mainly *N. tabacum* [3, 4, 14, 17, 18, 19] and its progenitor species *N. tomentosiformis* [13], and *N. sylvestris* [3]. There is also a report of the loci and their functionality in a few South American wild species including *N. langsdorffii* and *N. alata* [15].

In Australia, there are 26 wild species and subspecies of *Nicotiana*, all belonging to *Suaveolentes* section [20, 21]. Australian species of *Nicotiana* are mostly annuals, or in exceptionally good seasons they may survive for a second year. In southern parts there are a few short-lived perennial species [22, 23]. Prior to and since European settlement in Australia, these species have been used as smokeless tobacco products by Aboriginal populations in central Australia [22]. Both the *Nicotiana* spp. and the final tobacco mix (quid) are frequently referred to as 'pituri' [22, 23, 24, 25], although other names are also used including 'mingkulpa' [24, 25]. The pituri quid is a mix of usually sun- or fire- dried leaves and stems of wild *Nicotiana* to which burnt alkaline wood ash is added [22, 25, 26]; the quid is then masticated for a short time before being held in the oral cavity for an extended period. This practice is known as chewing. Central Australian Aboriginal women report widespread use of pituri in their communities, with chewing commencing at around the age of 5 years old [27]. Recent preliminary research exploring the health effects of chewing pituri during pregnancy demonstrated adverse maternal and perinatal outcomes [28].

Worldwide, despite the number of smokeless tobacco users being counted as more than 300 million, the use of smokeless tobacco products has received relatively little research attention compared with the use of smoked tobacco [29]. Of the research that has been conducted, a significant portion has focused upon the use of smokeless tobacco by men, with that research showing increased rates of oral, lip, pancreatic, and stomach cancer [29, 30]. Research examining the effects of smokeless tobacco use on women is limited and generally focused upon the gestational effects. That research demonstrates a range of adverse findings similar to those from the use of smoked tobacco in pregnancy including lower birthweight

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

neonates and increased stillbirths [31]. Inherent difficulties in smokeless tobacco research arise as a consequence of the number and variety of products used and the different admixtures and their administration practices [31].

Understanding the botanical principles that lead to adverse health outcomes is significant to informing the biological premise of the future smokeless tobacco health research agenda in both Australia and overseas. In Australia a range of species have been reported to be used for chewing, depending on their availability in the different geographic locations. The most preferred one is *N. gossei* [22, 23, 32], which has been shown to contain nornicotine and TSNAs in its dry leaves and demonstrated potential for cytotoxicity [33]. When not available, other species such as *N. excelsior*, *N. rosulata* subsp. *ingulba*, *N. goodspeedii*, *N. benthamiana* and *N. cavicola* might be used [22, 23, 32]. The alkaloid profile of *Nicotiana* species has been reported previously for 19 out of 26 of the recognised Australian taxa [1, 2], but there is no information on nicotine to nornicotine conversion and the molecular characteristics of the responsible locus in Australian *Nicotiana* species. This study aims to quantify the pyridine alkaloids in all Australian *Nicotiana* spp. and investigate the nicotine to nornicotine conversion.

2. Results

2.1. Plant growth

Plants were successfully grown for 24 out of the 26 recognised taxa, with more than one seedlot grown for four species (Table 1). In total, 73 seed lots were obtained for the 26 Australian species and subspecies. Not all of the received seed lots were viable and of high enough quality to result in successful germination. Despite our best attempts, germination and growth of *N. wuttkei* and *N. umbratica* seeds were not achieved because the seeds that we obtained were either not viable or grew into plants that were identified to be other species: *N. tabacum* for seeds labelled as *N. wuttkei,* and *N. amplexicaulis* instead of *N. umbratica*. Collection of fresh seeds was not possible because records showing the presence of *N. wuttkei* in a small remote area of northern Queensland are over 20 years old and it hasn't been seen there for many years, and *N. umbratica* has a very limited distribution in northern Western Australia.

2.2. Alkaloid profile

Fresh leaves of all 24 taxa contained the major alkaloids nicotine and nornicotine (Table 2). Anatabine and anabasine were also found in the majority of the taxa but myosmine and cotinine were not detected. Nicotine constitutes the major portion of total alkaloids in *N. gossei*, which tends to be the most important chewed species amongst Aboriginal pituri chewers. This was also the case for *N. excelsior* and *N. benthamiana*, which are amongst the preferred species for pituri chewing [22,

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Table 1. Seeds used for growing the *Nicotiana* species for this study, with the corresponding Australian state from where they were collected, contributor accession code, herbarium voucher number, collection date, viability (V) and germination (G). Herbarium vouchers that have been submitted and confirmed but not yet received a number are indicated, a dash (–) indicates that the information is unavailable, a star (*) indicates a seed lot derived from cultivated plants that were grown from this original seed collection.

Taxon	State	Accession	Voucher	Collection	% V	% G
N. amplexicaulis N.T.Burb	QLD	904998	NSW 234670	21/10/1990	60	80
	QLD	TS 298 (AusTRCF317546)	MEL 2396301	–	50	50
N. benthamiana Domin	WA	A109412	NT A0109412	18/07/2001	90	100
N. burbidgeae Symon	SA	DJD3167	Voucher confirmed	09/2015	20	20
N. cavicola N.T.Burb.	WA	951497	NSW 877057	03/08/1995	100	90
N. excelsior (J.Black) J.Black	SA	D194512/PKL24979	AD 246203	21/09/2009	50	30
N. forsteri Roem. & Schult.	QLD	BGQLD.0727	BRI AQ0840316	27/05/2010	50	50
N. goodspeedii H.-M.Wheeler	SA	DJD213	AD 192908	22/11/2005	100	100
N. gossei Domin	NT	D204093	NT D0204093	25/04/2011	100	100
	NT	971349	Identity confirmed	09/1997	90	80
N. heterantha Symon & Kenneally	WA	AusTRCF313551	MEL 2396300	–	60	30
N. maritima H. Wheeler	SA	MKJ140	AD 187318	15/11/2005	80	80
	SA	DJD3341	Voucher confirmed	07/01/2013	50	30
N. megalosiphon Van Heurck & Mull. Arg. ssp. *megalosiphon*	NSW	20101470	NSW 870550	08/11/2010	100	90

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Table 1. (*Continued*)

Taxon	State	Accession	Voucher	Collection	% V	% G
N. megalosiphon Van Heurck & Mull. Arg. ssp. *sessifolia* P. Horton	-	AusTRCF303829	MEL 2396303	–	30	20
*N. monoschizocarpa (*P.Horton) Symon & Lepschi	NT	From cultivated plants of AusTRCF303666	MELU D106501, D106502, D106503, D106504	09/1986*	50	50
N. occidentalis H.Wheeler ssp.*hesperis* (N.Burb.) P.Horton	WA	TS 341 (AusTRCF303767)	MEL 2396304	1960	82	70
N. occidentalis H.Wheeler ssp. *obliqua* N. Burb.	–	From cultivated plants of AusTRCF303779	MELU D106540	08/1956*	70	50
N. occidentalis H.Wheeler ssp. o*ccidentalis*	WA	L 3569 (AusTRCF303738)	MEL2396331	1959	30	20
N. rosulata (S.Moore) Domin ssp. *ingulba* (J.Black) P. Horton	NT	TS 75 (AusTRCF303907)	MEL 2396302	–	84	50
N. rosulata (S.Moore) Domin *ssp. rosulata*	WA	20070392	PERTH 7821336	20/07/2006	100	100
N. rotundifolia Lindley	WA	SW	MEL 2396305	08/2015	100	100
N. simulans N.Burb.	SA	RJB70944	AD 206232	12/03/2007	80	70
N. sp. *'Corunna'* Symon 17088	SA	From cultivated plants of SL23	MELU D106460, D106461, D106462, D106463, D106464	09/2005*	50	50
N. suaveolens H. Wheeler	SA	DJD1980	AD 239956	6/10/2010	100	100
N. truncata Symon	SA	TST1056	AD 250303	7/10/2010	50	40
N. velutina H.Wheeler	SA	DJD233	AD 192706	24/11/2005	70	60
	VIC	890225	NSW 213331	24/01/1989	60	40
	NSW	20101508	NSW 872306	15/11/2010	40	20

Table 2. Alkaloid content (mean ± se for 3 replicates) in freeze-dried fresh leaves of Australian *Nicotiana* spp. grown in a plant growth incubator under 16 h photoperiod with cool white fluorescent lamps, 23 °C day and 21 °C night temperature, constant 70–90% relative humidity. Plants were watered daily as needed. Conversion rate was defined using the formula: [nornicotine content/(nornicotine content + nicotine content)] x 100. Species with less than 10% conversion rate are regarded to be low or nonconverters, 10–50% are medium converters, and more than 50% conversion rate are assigned as high converters.

# of accessions used	Species	Alkaloid Concentrations (mg/g D.W.)						Conversion Status
		Nicotine	Nornicotine	Anatabine	Myosmine	Anabasine	Cotinine	
2	*N. amplexicaulis*	1.61 ± 0.44	0.23 ± 0.12	0.17 ± 0.15	N.D.	0.28 ± 0.29	N.D.	Medium
		5.09 ± 0.15	0.15 ± 0.05	0.05 ± 0.02	N.D.	0.05 ± 0.02	N.D.	Non-Low
1	*N. benthamiana*	2.29 ± 1.61	0.25 ± 0.05	0.14 ± 0.10	N.D.	0.41 ± 0.32	N.D.	Non-Low
1	*N. burbidgeae*	4.05 ± 1.12	0.20 ± 0.09	0.01 ± 0.00	N.D.	0.66 ± 0.20	N.D.	Non-Low
1	*N. cavicola*	0.06 ± 0.02	0.10 ± 0.02	BLQ	N.D.	0.01 ± 0.02	N.D.	High
1	*N. excelsior*	5.39 ± 1.09	0.17 ± 0.14	0.21 ± 0.05	N.D.	0.17 ± 0.10	N.D.	Non-Low
1	*N. forsteri*	1.39 ± 0.35	0.66 ± 0.47	0.24 ± 0.04	N.D.	0.74 ± 0.5	N.D.	Medium
1	*N. goodspeedii*	0.24 ± 0.18	1.92 ± 0.08	0.03 ± 0.01	N.D.	0.29 ± 0.11	N.D.	High
2	*N. gossei*	5.95 ± 1.33	0.05 ± 0.07	0.07 ± 0.02	N.D.	N.D.	N.D.	Non-Low
		8.27 ± 3.00	0.05 ± 0.02	0.11 ± 0.06	N.D.	0.10 ± 0.04	N.D.	Non-Low
1	*N. heterantha*	0.89 ± 0.12	0.19 ± 0.11	BLQ	N.D.	0.48 ± 0.11	N.D.	Medium
2	*N. maritima*	3.94 ± 1.39	0.12 ± 0.05	0.05 ± 0.02	N.D.	0.11 ± 0.05	N.D.	Non-Low
		3.17 ± 1.93	0.10 ± 0.06	0.04 ± 0.02	N.D.	0.11 ± 0.07	N.D.	Non-Low
1	*N. megalosiphon* subsp. *megalosiphon*	0.11 ± 0.07	0.63 ± 0.2	0.03 ± 0.01	N.D.	0.23 ± 0.12	N.D.	High
1	*N. megalosiphon* subsp. *sessifolia*	0.14 ± 0.08	0.76 ± 0.30	0.11 ± 0.06	N.D.	0.03 ± 0.01	N.D.	High
1	*N. monoschizocarpa*	2.35 ± 0.38	2.82 ± 0.66	0.14 ± 0.12	N.D.	2.67 ± 0.83	N.D.	High
1	*N. occidentalis* subsp. *hesperis*	0.41 ± 0.01	0.14 ± 0.10	0.03 ± 0.01	N.D.	0.17 ± 0.02	N.D.	Medium
1	*N. occidentalis* subsp. *obliqua*	0.65 ± 0.32	0.29 ± 0.04	0.10 ± 0.07	N.D.	0.47 ± 0.45	N.D.	Medium
1	*N. occidentalis* subsp. *occidentalis*	0.15 ± 0.06	0.47 ± 0.12	BLQ	N.D.	0.10 ± 0.00	N.D.	High
1	*N. rosulata* subsp. *rosulata*	0.60 ± 0.04	0.10 ± 0.03	0.19 ± 0.11	N.D.	0.13 ± 0.04	N.D.	Medium

(*Continued*)

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

Table 2. (*Continued*)

# of accessions used	Species	Alkaloid Concentrations (mg/g D.W.)						Conversion Status
		Nicotine	Nornicotine	Anatabine	Myosmine	Anabasine	Cotinine	
1	*N. rosulata* var. *ingulba*	0.81 ± 0.06	0.18 ± 0.08	0.02 ± 0.01	N.D.	0.01 ± 0.02	N.D.	Medium
1	*N. rotundifolia*	3.54 ± 1.58	0.15 ± 0.17	0.02 ± 0.03	N.D.	0.24 ± 0.04	N.D.	Non-Low
1	*N. simulans*	0.04 ± 0.04	0.44 ± 0.04	0.03 ± 0.00	N.D.	0.10 ± 0.02	N.D.	High
1	*N. sp. 'Corunna'*	1.61 ± 1.10	0.07 ± 0.03	0.04 ± 0.02	N.D.	0.25 ± 0.12	N.D.	Non-Low
1	*N. suaveolens*	0.15 ± 0.21	0.72 ± 0.35	0.01 ± 0.01	N.D.	0.12 ± 0.06	N.D.	High
1	*N. truncata*	3.21 ± 0.46	0.11 ± 0.07	0.23 ± 0.12	N.D.	0.11 ± 0.03	N.D.	Non-Low
3	*N. velutina*	0.11 ± 0.06	0.92 ± 0.53	0.05 ± 0.06	N.D.	0.31 ± 0.25	N.D.	High
		0.03 ± 0.03	1.18 ± 0.28	BLQ	N.D.	0.31 ± 0.03	N.D.	High
		0.16 ± 0.03	0.22 ± 0.06	BLQ	N.D.	0.23 ± 0.03	N.D.	High

N.D.: Not Detected.

BLQ: Below Limit of Quantification.

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32]. Hence, these three species are examples of low or nonconverter phenotypes. In contrast, nornicotine is the predominant alkaloid in *N. goodspeedii*, *N. velutina* and *N. cavicola* and so these are classified as high converter species. For all of the species for which more than one seed sources were available for comparison (*N. amplexicaulis*, *N. gossei, N. maritima* and *N. velutina*) the dominant alkaloid was the same and absolute concentrations were not significantly different (Table 2).

Treatment with heat to mimic curing elevated the proportion of nornicotine to nicotine in the leaves of both high converter and low or nonconverter species. However this increase did not change the dominant alkaloid in either group (Table 3).

2.3. CYP82E genes in Australian *Nicotiana* spp.

Since nicotine N-demethylase (NND), the main source for conversion of nicotine to nornicotine, is encoded by cytochrome P450 monooxygenase genes of the CYP82E subfamily in *N. tabacum* [4, 17], PCR using CYP82E specific primers based on the sequences of the conserved regions of the subfamily in *N. tabacum* (Table 4) was optimised to amplify the potential loci in Australian *Nicotiana* species. Based on the agarose gel electrophoresis results, genomic DNA corresponding to the conserved region of CYP82E related genes have been amplified in all of the studied Australian *Nicotiana* species (Fig. 1). To confirm the identity, the amplified regions from three low or nonconverter and three high converter species were purified and subjected to Sanger sequencing. The DNA sequences of the CYP82E related genes from *N. benthamiana*, *N. gossei*, *N. goodspeedii*, *N. velutina*, *N. cavicola* and *N. excelsior* have been deposited in NCBI GenBank with the accession numbers KU234094, KU234095, KU234096, KU234097, KU234098 and KU504631, respectively. Blasting the obtained

Table 3. Alkaloids (mean ± se for 3 replicates) of fresh versus cured (35 °C for 6 h in the dark) leaves for selected species of Australian *Nicotiana* spp. Significant differences ($p < 0.05$) between fresh and cured leaves are indicated (*).

Species	% Nornicotine content/(Nicotine content + Nornicotine content)	
	Fresh leaves	Cured leaves
N. benthamiana	9.6 ± 2.0	21.4 ± 3.1*
N. excelsior	2.9 ± 0.9	6.0 ± 2.6
N. gossei	0.4 ± 0.1	0.7 ± 0.2
N. goodspeedii	83.2 ± 1.1	90.5 ± 0.8*
N. cavicola	69.7 ± 1.6	81.4 ± 2.6*
N. velutina	73.3 ± 3.8	92.6 ± 1.7*

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

Table 4. Sequences of the primers used for PCR amplification of CYP82E related genes designed from *N. tabacum* CYP82E4 mRNA (Accession: KC120817.1) and internal gene GAPDH designed from *N. benthamiana* glyderaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Accession: JQ256517.1).

Primers	Sequence (5'–>3')	Product length	Comment
CYP82E-F	CTGCGGACACAGTTGCTCTT	536	For amplifying CYP82E subfamily genes from genomic DNA and cDNA
CYP82E-R	AGTTATGCCTGCACCTTCCT		
GAPDH-F	AACCGGTGTCTTCACTGACAAGGA	562	For amplifying internal gene GAPDH from cDNA
GAPDH-R	GCTTGACCTGCTGTCACCAACAAA		

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

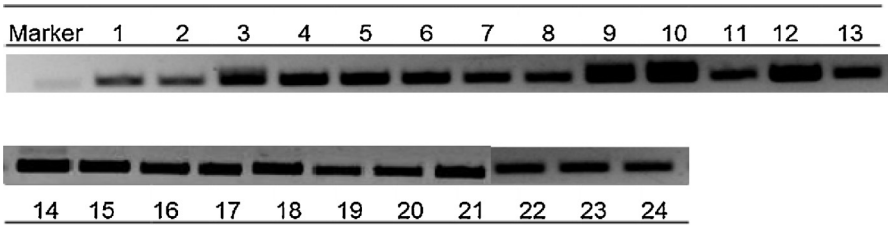

Fig. 1. Image of the agarose gel electrophoretis for the CYP82E related fragment. The marker shows the part of the DNA ladder corresponding to 0.5 kb. Fragments were produced by 35 cycles of PCR amplification of DNA isolated from 1. *N. forsteri,* 2. *N. benthamiana,* 3. *N. excelsior,* 4. *N. gossei,* 5. *N. suaveolens (exigua),* 6. *N. goodspeedii,* 7. *N. simulans,* 8. *N. velutina,* 9. *N. cavicola,* 10. *N. amplexicaulis,* 11. *N. megalosiphon subsp. Megalosiphon,* 12. *N. megalosiphon subsp. sessifolia,* 13. *N. truncate,* 14. *N. maritima,* 15. *N. occidentalis subsp. obliqua,* 16. *N. occidentalis subsp. occidentalis,* 17. *N. occidentalis subsp. hesperis,* 18. *N. sp. 'Corunna',* 19. *N. heterantha,* 20. *N. rosulata subsp. rosulata,* 21. *N. rosulata var. ingulba,* 22. *N. rotundifolia,* 23. *N. burbidgeae,* 24. *N. monoschizocarpa.* (Full gel Images are provided as supplementary files: "Supplementary file-Gel Image- Fig. 1-1 to 1-3").

sequences in NCBI BLASTN tool demonstrated 94–97% resemblance and identity to the published sequences of conversion locus CYP82E in other *Nicotiana* species, confirming their proposed identity (DQ131887.2 and EF472002.1).

2.4. Agarose gel electrophoretic analysis of CYP82E related genes transcript levels in fresh leaves versus leaves treated with simulated curing in selected species

The relationship between transcript levels of CYP82E related genes and observed levels of nornicotine production in fresh leaves with those from leaves treated with simulated curing in high converter and low or nonconverter species was investigated by analysing relative transcript accumulation levels in fresh versus cured leaves. The ratio between the optical density of the CYP82E band to that from the GAPDH band on the agarose gel photo for each sample was used as an indicator of expression and calculated in fresh and cured leaves of both high converter and low or nonconverter groups of species. The normalised optical density of the bands resulting from transcription of the CYP82E related genes in fresh leaves showed no significant difference across high converter and low or nonconverter species. Simulated curing was associated with a significantly higher (almost 3 fold) optical density in the leaves of the high converter species, but only a small increase or no change was measured in the cured leaves for the low or nonconverter species (Fig. 2).

3. Discussion

This study has expanded the range of Australian wild *Nicotiana* species and subspecies assessed for alkaloid composition to 24 out of the 26 that are currently recognised (Table 2). The taxa missing from analysis are *N. wuttkei* and *N.*

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

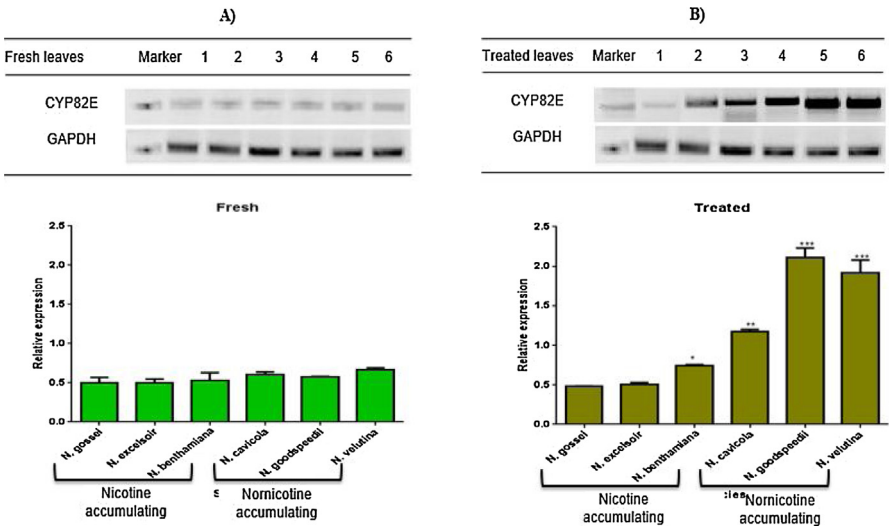

Fig. 2. Agarose gel electrophoretic analysis and normalised band densitometry values of the CYP82E related fragment. The marker shows the part of the DNA ladder corresponding to 0.5 kb. The fragment was produced by 35 cycles of PCR amplification of cDNA isolated from A) fresh leaves and B) cured leaves (at 35 °C for 6 h) of 1. *N. gossei* 2. *N. excelsior* 3. *N. benthamiana,* 4. *N. cavicola,* 5. *N. goodspeedii,* 6. *N. velutina.* A 562 bp GAPDH fragment obtained by 35 cycles of PCR amplification of the cDNA for each sample is shown as an internal gene and for normalising the densitometric values that all were quantified using ImageJ software. The data points in graphs show mean ± std error of 3 independent experiments, * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$. (Full gel images are provided as supplementary files: "Supplementary file-Gel Image- Fig. 2A and B").

umbratica for which we were unable to obtain a reliable seed source. Alkaloid composition exhibited large differences between species, with a maximum of 8.3 and minimum of 0.3 mg/g nicotine recorded, which were for *N. gossei* and *N. velutina*, respectively. Alkaloid production in *Nicotiana* is controlled genetically and levels can vary dramatically throughout growth in response to environmental conditions [34] so it may be expected that different seedlots of the same species vary in alkaloid quantities even when grown in a single environment. However, four species for which two or three seed sources from different geographic locations within Australia were grown (*N. amplexicaulis*, *N. gossei, N. maritima*, and *N. velutina*), showed very little difference in alkaloid concentrations. The alkaloid composition for 19 Australian *Nicotiana* species were reported almost 30 years ago [1, 2]. Differences in absolute alkaloid concentrations to those reported here may be associated not only with the different seedlots involved, but also differences in the growth environment and treatment of plants before harvesting leaves. The plants used in previous studies were grown in non-Australian greenhouse [1, 2] or field [1] environments, whereas the plants herein were grown in a laboratory; usually laboratory-grown plants contain lower alkaloid concentrations than those from field or greenhouse [35]. Despite differences in reported absolute concentrations for nicotine and nornicotine between this and previous

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studies, the inferred conversion trait for each species is consistent between studies. For example, using the nicotine and nornicotine quantification results reported by both previous studies [1, 2] and here (Table 2), the species *N. gossei, N. excelsior* and *N. benthamiana* are categorised as low or nonconverter, while *N. goodspeedii, N. cavicola* and *N. velutina* are high converter species.

Nornicotine is an undesirable alkaloid in consumed tobacco species because its production and accumulation has a positive correlation to production of the carcinogenic nitrosamine, NNN. It is notable that the main species preferred for chewing are amongst those that mainly accumulate nicotine, i.e. *N. gossei* and *N. excelsior,* both of which are low or nonconverters. *N. benthamiana*, a low converter, has also been mentioned to be used for chewing in Western Australia [22, 32]. In contrast, the high converters *N. megalosiphon* ssp. *megalosiphon, N. simulans, N. occidentalis* ssp. *occidentalis* and *N. velutina* have been listed in the literature as being rarely used or specifically avoided [22, 23, 32]. However, the high converter *N. cavicola* has been mentioned as being chewed in Western Australia [32], and tops and roots of high converter *N. goodspeedii* have been reported to be used by Aboriginal people at the eastern end of the Nullarbor Plain and to the south [32]. *N. rosulata* ssp. *ingulba* is reportedly a species that is preferred for chewing due to its availability in central Australia [22, 32, 36], but this is a medium converter.

Production of nornicotine via N-demethylation of nicotine in commercial tobacco is mainly governed by CYP82E related genes, specifically E4 [37, 38]. Given the vital importance of the CYP82E related genes in nornicotine production and accumulation in tobacco plants, and due to considerable nornicotine production in some Australian *Nicotiana* species and the unknown source of conversion in them, in this study we amplified the conversion loci in all 24 Australian *Nicotiana* species and subspecies. This is the first molecular and biochemical investigation of the CYP82E genes in Australian *Nicotiana* species. Even though the taxa clearly differ in their conversion of nicotine to nornicotine, the locus was present in all studied taxa. The difference in the observed nicotine to nornicotine conversion phenotype could be due to the presence of the CYP82E related genes with different ability for encoding functional nicotine N-demethylases.

Due to the large number of species and the little information on the conversion locus in species other than *N. tabacum*, the varieties and number of different genes have not been identified in detail in this study. Instead, the relative functionality of the loci has been compared for six selected high converters and low or nonconverter species. The functional CYP82E loci, especially E4, have been reported to be triggered by curing which leads to a high level of transcript accumulation only in high converter species, while the transcript accumulation happens in very low and negligible levels in low or nonconverters [17]. This could

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be an explanation for the observed significantly elevated levels of transcripts (3–4 fold) in the cured leaves of only high converter species *N. goodspeedii*, *N. cavicola* and *N. velutina*, while no significant difference was found between the level of transcript accumulation in fresh leaves with the fresh and cured leaves of the low or nonconverter species *N. gossei* and *N. excelsior*. The level of transcripts in cured leaves of *N. benthamiana* shows a significant increase compared to that observed in fresh leaves, although the increase was smaller than observed for the high converters. This is also consistent with the conversion phenotype of *N. benthamiana*, which although it accumulates nicotine as the most abundant alkaloid, it tends to accumulate higher nornicotine in cured leaves compared to that observed for the other two low or nonconverter species. This might be due to presence of the minor functional NNDs in *N. benthamiana*. The alkaloid phenotypes of the two low or nonconverters, *N. gossei* and *N. excelsior*, show a lower proportion of nornicotine to nicotine in fresh and cured leaves with a very small increase after curing that is also consistent with the illustrated transcript accumulation levels. This might be due to the loci present in these species being either non-functional or with minor functionality and expressed only at a basic steady level. In contrast, the loci in high converter species, *N. goodspeedii*, *N. cavicola* and *N. velutina*, are functional and triggered to increase their transcript levels, and this in turn is responsible for their higher levels of nornicotine.

The CYP82E subfamily has several genes in *N. tabacum* and other studied *Nicotiana* species that share a high level of sequence identity (in some cases more than 90%), however the majority of these are not functional NND genes [17]. There are also genes that are functional in some species, but have been mutated and become non-functional in others [3]. This could be due to either transcriptional inactivation or premature translational termination of these genes, resulting in the absence of nornicotine in their carrying species. For example, *N. tabacum* is the result of hybrizidation of diploid *Nicotiana* species closely related to modern *N. tomentosiformis* and *N. sylvestris. N. tomentosiformis* has both active CYP82E3 and CYP82E4 which control conversion in green and senescing leaves, respectively. In contrast, in *N. tabacum* a W330C amino acid substitution inactivates CYP82E3; moreover, in nicotine-accumulating nonconverter *N. tabacum* a CYP82E4 is also transcriptionally silenced [39]. Another example is in the two closely related wild species of *Alatae* section, *N. langsdorffii* and *N. alata*. In *N. langsdorffii* the CYP82E genes are rendered non-functional, whereas CYP82E genes in *N. alata* act semi-dominantly and individually to increase conversion of nicotine to nornicotine [15]. Furthermore, the tobacco genome contains numerous pseudogenes with CYP82E-like sequences that have been mutated [15]. CYP82E4 has been reported to be the major functional NND gene, while there are other genes such as CYP8210 and CYP82E5v2 within the *N. tabacum* genome that are also functional, but result in lower level of conversion

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and hence are considered to be minor functional NNDs. This study took the first step in investigating the underlying mechanism in nicotine to nornicotine conversion in Australian *Nicotiana*. As only part of the coding sequence is amplified (536 bp out of approx 1,500 bp), and since previous studies have identified numerous point mutations in this isoform that can render it inactive, further phylogenetic studies to establish orthology with the CYP82E4 isoform as well as yeast expression studies will be needed to confirm the NND activity of the amplified sequence.

4. Conclusion

In conclusion, the nicotine to nornicotine conversion rate varies between *Nicotiana* species from the *Sauveolentes* section. The conversion locus was studied in 24 Australian taxa, but the functional NND enzymes might be active only in nornicotine accumulating species. This seems to be driven mainly by their genetic capability rather than environmental factors [35], so even in the species with more than one seed source from different environmental conditions the conversion trait is the same. The observed high nicotine to nornicotine converter phenotype in some Australian *Nicotiana* species poses a potential risk of toxicity to chewers of these species, especially if the leaves are processed and cured before being used in the preparation of pituri, as nornicotine and its nitrosamine, NNN, can be detrimental to health. Given the extent of pituri use by central Australian Aboriginal populations, further combined botanical and health research is important in developing the evidence base from which appropriate health literacy information can be developed.

5. Materials and methods

5.1. Plant material

There are 26 recognised taxa of Australian *Nicotiana* [20, 21]. Attempts were made to obtain seeds of these 26 species and subspecies from seedbanks, botanic gardens and herbaria across Australia (for full information on the seed lots used in the course of this study refer to the supplementary table). Seed viability for each seedlot was determined with tetrazolium staining. Seeds were imbibed in distilled water for 24 h and then incubated on filter paper soaked with 1% 2,3,5-triphenyltetrazolium chloride (Sigma) solution for 24 h at 35 °C in darkness. The viability was determined by scoring the embryos stained red or pink as viable and the number that were viable was calculated as a % of those tested. For germination, 10 seeds for each seedlot were incubated in 55 mm plastic Petri dishes on a double layer of filter paper (Advantec NO.1 55 mm) soaked with 5 mL distilled water containing 200 ppm GA3 (Sigma) at 20 °C under 16 h photoperiod with cool white light. After 24 h seeds were transferred to Petri dishes with filter papers soaked in only distilled water. A seed was considered

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to have germinated once the radicle emerged, and the number of seeds that germinated was calculated as a % of those on the plate. After 10–14 days, germinated seeds were transferred into seedling trays filled with Osmocote seed raising and cutting mix (Scotts, Australia). After 2 weeks seedlings were moved to 100 × 50 mm black tube pots containing Osmocote native potting & planting mix (Scotts, Australia). Plants were grown in pots and under controlled environment (Thermoline, Climatron 520 & 1100-DL/SL growth cabinet) with 16 h light provided by cool white fluorescent lamps, 23 °C day and 21 °C night temperature, constant 70–90% relative humidity and watered daily as needed. For seedlots with low seed viability, this process was repeated until 3 healthy plants were obtained.

Green leaves were harvested from 8–10 week old non-flowering adult plants. Three plants were sampled for each taxon, forming the three separate replicates for all subsequent analyses. Fresh leaf material was used for DNA extraction for CYP82E gene analysis. Fresh leaves were freeze-dried and stored at −80 °C for alkaloid analysis.

Six species were selected based on results of alkaloid analysis for more detailed investigations of the conversion locus: three low or nonconverter species (*N. benthamiana, N. excelsior, N. gossei*) and three high converter species (*N. cavicola, N. goodspeedii, N. velutina*). The seedlots used for *N. gossei* and *N. velutina* were those listed first in Table 1. A short heat treatment was used to provide an indication of the changes that might occur in cured leaves. Fresh leaves were cut in half and one half was directly extracted for RNA and freeze-dried for alkaloids analysis. The other half was treated with a simulated curing condition by incubating at 35 °C for 6 h in the dark and then used for RNA extraction for RT-PCR analysis and freeze-dried for alkaloid analysis.

Herbarium vouchers were prepared and submitted using the laboratory-grown plants unless a record for the specific seedlot already existed; the confirmation of identity for six taxa was performed by Neville Walsh (Senior Conservation Botanist, Royal Botanic Gardens Victoria).

5.2. Chemical analysis

5.2.1. Chemicals and reagents

The solvents used for alkaloids extraction and HPLC analysis were methanol and acetonitrile, from Merck (Darmstadt, Germany), and a 15 mM ammonium formate buffer prepared using ammonium formate from Sigma-Aldrich (St. Louis, MO). The water was deionised and filtered using a Milli-Q system (Millipore, Billerica, MA). Hydrochloric acid and sodium hydroxide used for adjusting pH were from Merck (Darmstadt, Germany). The analytical standards used were nornicotine, myosmine and cotinine from Sigma, nicotine from Fluka (Milwaukee, WI),

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anabasine from Sigma-Aldrich, anatabine from Cayman Chemical Company (AnnArbor, MI), and the internal standards used were caffeine from Ajax Finechem (Sydney, Australia).

5.2.2. Alkaloid analysis

Both fresh and cured freeze-dried leaves (50 mg) were exhaustively extracted for pyridine alkaloids using 40% aqueous methanol containing 0.1% 1 N hydrochloric acid following our previously published method [40] and were quantified mainly for nicotine, nornicotine, anatabine and anabasine. Myosmine and cotinine that have been mentioned to be present in some tobacco plants [41, 42, 43] were also quantified in the studied plants. Quantitative analysis of the extracts was carried out on an Agilent 1100 series high performance liquid chromatography (HPLC) system equipped with a UV detector and on a Zorbax Extend C18 column (Agilent Technologies, Mulgrave, Vic, Australia) with a gradient mobile phase consisting of 15 mM ammonium formate buffer and acetonitrile [40]. Percent conversion was calculated as [nornicotine content/(nornicotine content + nicotine content)] x 100 [15]. Plants with less than 10% were scored as low or nonconverters, plants with 10–50% and 50–100% conversion were assigned as medium and high converters, respectively.

5.3. Molecular analysis

5.3.1. PCR amplification of CYP82E related genes

Genomic DNA was extracted from fresh leaves using the ZR Plant/Seed DNA MiniPrep^{TM} (Zymo Research, CA, USA) following the manufacturer's instructions. Genomic fragments containing CYP82E related genes were obtained by amplifying genomic DNA (around 20 ng) with PCR primer set that was designed based on the conserved sequences of the published tobacco CYP82E subfamily genes (Table 4). Gel electrophoresis was then performed by loading 5 μl of the PCR products to investigate the presence of the bands representing the amplified targets in studied samples. A 1 kb DNA ladder (New England Biolabs Inc, MA, USA) was used as marker.

5.3.2. Sequencing the amplified CYP82E regions for selected species

The amplified genomic DNA from the three selected low or nonconverter species (*N. benthamiana, N. excelsior, N. gossei*) and three high converter species (*N. cavicola, N. goodspeedii, N. velutina*) were purified using Rapid PCR Cleanup Enzyme Set (New England Biolabs Inc, MA, U.S.A) according to the protocol of the manufacturer. Each purified PCR product was sent for BDT labelling, purification and sequencing to the Australian Genome Research Facility and

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sequenced following high throughput Sanger sequencing using Applied Biosystems 3730 and 3730xl capillary sequencers. These automated platforms use ABI Prism BigDye Terminator kit (BDT) chemistry version 3.1 (Applied Biosystems) under standardised cycling PCR conditions. cDNA sequences of CYP82E subunits were retrieved from GenBank and BLASTn was used for confirmation of the identity of CYP82E subunits.

5.3.3. Agarose gel electrophoretic analysis of CYP82E related genes transcript levels in fresh versus cured leaves of selected species

Total RNA was extracted from fresh and cured leaves of three low or nonconverter species (*N. benthamiana, N. excelsior, N. gossei*) and three high converter species (*N. cavicola, N. goodspeedii, N. velutina*) using the ZR Plant RNA MiniPrep™ (Zymo Research, CA, USA) following the instructions provided by the manufacturer. First strand cDNA was then synthesized by ProtoScript II First Strand cDNA Synthesis Kit (New England biolabs Inc, MA, U.S.A) according to the protocol of the manufacturer. The CYP82E related cDNA fragments were amplified by PCR amplification of isolated cDNA from the fresh and cured leaves of the studied species. A set of primers was also optimised to amplify 562 bp GAPDH fragments from the obtained cDNA to be used as the internal gene (Table 4). Optical densitometric analysis from the picture of the obtained agarose gel electrophoresis bands was then performed using ImageJ software (version 1.48b, https://imagej.nih.gov/ij/) to determine the relative gene expression of the CYP82E related genes in fresh versus cured leaves. The optical density of the CYP82E bands obtained from loading 5 μl of the PCR product was normalised to that of GAPDH bands obtained from loading similar amount of 5 μl of the PCR product used as internal gene.

5.4. Statistical analysis

Statistical analysis was performed using Prism (GraphPad, San Diego, CA) software. One-way analysis of variance (ANOVA) was applied with Bonferroni multiple comparisons and $p < 0.05$ marked as significant. ImageJ Software version 1.48b (https://imagej.nih.gov/ij/) was used for the optical densitometric analysis of expression levels in fresh versus cured leaves.

Declarations

Author contribution statement

Nahid Moghbel: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

http://dx.doi.org/10.1016/j.heliyon.2017.e00469

BoMi Ryu, Kathryn Steadman: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Angela Ratsch: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Additional information

Supplementary content related to this article has been published online at http://dx.doi.org/10.1016/j.heliyon.2017.e00469

Acknowledgments

Many thanks to those who collected and donated seeds for this study, especially Daniel Duval (South Australian Seed Conservation Centre, SA), Graeme Errington (Australian Plantbank, NSW), Brenda Leighton (Australian Tropical Grains Germplasm Centre, Qld), Steve Wylie (WA State Agricultural Biotechnology Centre, WA), Philip Cameron (Brisbane Botanic Gardens, Qld), Gary Dinham (Alice Springs Desert Park, Parks and Wildlife Commission, NT), Megan Hirst (Royal Botanic Gardens Victoria), Ed Newbigin (School of BioSciences, The University of Melbourne, Vic), Shane Turner (Botanic Gardens and Parks Authority, WA). Special thanks to Dr Neville Walsh (Royal Botanic Gardens Victoria) who has been a great help in identifying some of the *Nicotiana* species using voucher specimens prepared from the studied plants. We thank Prof Jiahu Xie at North Carolina Central University and Dr Iman Azimi at the University of Queensland for their expert advice for this study.

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