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Short Communication

Screening a diverse collection of *Artemisia annua* germplasm accessions for the antimalarial compound, artemisinin

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Abstract

The antimalarial drug artemisinin (ART) is commercially extracted from the medicinal plant *Artemisia annua* L. Here, we report the screening of 70 *A. annua* plants representing 14 diverse germplasm accessions sourced from around the world, and identify lines containing > 2% ART. These extremely high-yielding individuals have been maintained as vegetative clones, and they represent promising germplasm resources for future *A. annua* breeding programmes.

Keywords: chemovar; plant pre-breeding; *Plasmodium falciparum*

Experimental methods

Malaria, a vector-borne disease caused by *Plasmodium falciparum*, infects 500 million people annually, predominately in sub-Saharan Africa (Snow *et al*., 2005). Resistance of *P. falciparum* to the widely used antimalarial drug chloroquinone means that this control approach is now ineffective in most malarial regions (Talisuna *et al*., 2007). Accordingly, the World Health Organization (WHO) has recommended administration of artemisinin (ART) in combination with other antimalarial drugs (artemisinin-based combination therapy, ACT) in chloroquinone-resistant regions (WHO, 2000). Artemisinin is a sesquiterpene lactone endoperoxide extracted from the predominantly outbreeding medicinal plant *Artemisia annua* L. ($2n = 18$). Although ART biosynthesis has not yet been fully elucidated, it involves the cytosolic mevalonate and plastid-located 1-deoxyxylulose pathways (Covello *et al*., 2007; Towler and Weathers, 2007). As ART cannot currently be economically synthesized using bulk fermentation or chemical synthesis (Van Noorden, 2010), extraction from plants remains the most

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economically feasible method. However, commercial supply of ART has historically fluctuated due to limitations in the quality of available plant material, climatic conditions and agricultural management. Concentration of ART in surveys of *A. annua* accessions is known to be predominantly <1%, with the highest content reported being 1.4% (Woerdenbag *et al.*, 1994; Delabays *et al.*, 2001; Kumar *et al.*, 2004; Khanuja *et al.*, 2005). As part of a wider programme aimed at enhancing ART concentration and supply (Davies *et al.*, 2009), we report the screening of a collection of 70 *A. annua* plants sampled from 14 diverse (and genetically heterogeneous) germplasm accessions, identifying germplasm with ART concentrations >2.0%.

Fourteen *A. annua* accessions from a range of geographical locations were collated (Table 1). For each accession, five plants were germinated in a heated glasshouse, and transplanted into field nurseries (45 × 45 cm spacing, single individuals from each population arranged within each of five blocks, following a randomized block design) on 30th April 2010 (Cambridge, UK, 52.20°N, 0.13°E). Flowering time (date on which the first individual within each accession flowered) and overall vegetative vigour (1: very vigorous, 2: vigorous, 3: weak, 4: heterogeneous) were scored. Additionally, ART concentrations (% wt/dry wt) were determined for each individual at harvest: material harvested from longitudinal sections through each plant was collected on 19th September and dried at 40°C for 24 h in a fan-assisted oven. Solid-phase extraction from dried leaf material was performed by sonication with analytical grade hexane (Fisher Scientific Ltd, Loughborough, UK). Aliquots of the supernatants were evaporated under N_2 gas flow and the dry extract reconstituted into mobile phase (1 ml) prior to injection. ART concentration was analysed using an Agilent 1100 HPLC equipped with a Phenomenex Luna C18 column (250 mm × 4.6 mm, particle size 5 μm, pore size 100 Å) and a UV DAD detector (215 nm), mobile phase = acetonitrile/water (60/40, v/v) at a mobile phase flow rate of 1 ml/min, column oven temperature: 25°C. Calibration of the HPLC method was determined using 10 mcg/ml of pure artemisinin standard (purity >98%; confirmed by melting range, found to be 150–152°C) every tenth sample during the determination of the investigated samples, returning coefficients of variation of 1%. Exemplar individuals from each accession are maintained at NIAB by vegetative propagation (Waseem *et al.*, 2011).

All but two accessions (1001 and 1012) flowered when grown in the field under UK summer day lengths (maximum: 16 h 48 mins, 21st June; minimum: 12 h 28 mins, 19th September). Mean ART concentration within the 14 accessions was found to vary from 0.08% (accession 1014) to 2.03% (accession 1015) (Table 1), with calibration controls of pure artemisinin standards returning coefficients of variation of ~1%. Intra-accession standard deviations (SDs) ranged from 0.02 to 0.64, with individual 1015-4 displaying the highest ART concentration (2.22%). Indeed, all five individuals from accession 1015 displayed elevated levels of ART, resulting in a high mean (2.03% ART) and a low SD (0.19). The only other accession with mean ART concentration >1% was 1013 (mean 1.45, SD 0.64), including an individual with an ART concentration of 2.16 (1013-4). The highest-yielding

Table 1. Artemisinin (ART) concentration, vegetative vigour and flowering time scores in the *Artemisia annua* accessions investigated

Code	Supplier/source	Mean % ART[a]	SD[a]	Max % ART (line)[a]	Vigour[b]	Flowering[c]
1001	Private collection	0.73	0.24	1.02 (1001-5)	2	N
1003	Seedman	0.79	0.15	0.95 (1003-5)	4	Y
1004	Pintree garden seeds	0.40	0.29	0.89 (1004-1)	2	Y
1006	Kettleby herb farm	0.18	0.05	0.26 (1006-5)	4	Y
1007	Variety, Qing Yao	0.19	0.02	0.21 (1007-3)	2	Y
1008	Sweet Annie seed	0.30	0.16	0.59 (1008-1)	2	Y
1012	German herb collection	0.93	0.42	1.5 (1012-4)	4	N
1013	Rich farm garden	1.45	0.64	2.16 (1013-4)	2	Y
1014	Cramer's Yardstick	0.08	0.02	0.1 (1014-2)	3	Y
1015	Private collection	2.03	0.19	2.22 (1014-4)	2	Y
1016	Moutcadi Reixac	0.50	0.18	0.79 (1016-3)	1	Y
1018	J. Mathews, 27-11-1996, France	0.37	0.08	0.5 (1018-2)	1	Y
1019	A. Susanna, XI-1999, Uzbekistan	0.57	0.56	1.25 (1019-3)	2	Y
1023	Montcadi Reixax 2-1-1997, Spain	0.14	0.03	0.18 (1023-1)	2	Y

SD, standard deviation.
[a] Mean ART concentration and SD of five individual plants per accession are shown, as well as the individual line with the highest ART concentration for each accession. ART is measured as % of dry weight. [b] Vegetative vigour: 1 (very strong growth), 2 (strong growth), 3 (weak growth), 4 (segregating for growth type). [c] Flowering in the field: Y (flowering), N (no flowering).

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accession (1015) was grown in the field the following year, included within a replicated block design, returning a mean ART concentration of 2.10, indicating consistently high ART yields across the two growth seasons investigated. Evaluation of overall growth type (Table 1) found just two accessions (1016, 1017) to possess very vigorous growth habit (score 1); both displayed relatively low mean ART concentrations ≤0.5 (Table 1). However, high-yielding ART lines 1013 and 1015 both possessed good (score 2) growth habits.

Discussion

Despite increasing deployment of ACT, and the recent development of genomic tools in *A. annua* (Graham *et al.*, 2010), comparatively little systematic evaluation has been reported in *A. annua* germplasm. Here, we screen a wide range of *A. annua* lines for ART concentration and agronomic suitability to European cultivation. The finding that the majority of accessions flowered under UK summer photoperiods indicates that the previously reported requirement for short-day photoperiods (~8 h) for floral initiation is not absolute, or that the critical minimum day length is higher than previously assumed. The competence to flower under UK conditions demonstrates that future breeding activities can be conducted without the need to artificially reduce photoperiod. Considerable variation in ART concentration was found within accessions. Previous studies show that ART concentration is a highly heritable trait (Delabays *et al.*, 2001). However, due to the out-breeding nature of *A. annua,* considerable intra-accession genetic variability is predicted to be present. Accordingly, ART concentrations in *A. annua* are reported to vary wildly, ranging between 0.03 and 1.4% of dry weight. Here, we identify *A. annua* lines combining good growth habit (score 2) and competence to flower under long-day photoperiods, with ART concentrations in excess of 2.0%, providing enhanced chemovars from which improved parental lines and commercial F_1 hybrid seeds can be developed. The establishment of improved breeding lines forms the basis from which future genetic and genomic studies can be best undertaken. Immortal lines for all accessions are maintained at NIAB (http://www.niab.com/) by vegetative propagation.

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