Geographical variation of *Juglans regia* L. in juglone content: rapid analysis using micro-plate reader

Juglans regia L. is a monoecious, heterodichogamous, deciduous tree species valued for its high-quality timber and its nuts[1]. It belongs to the family Juglandaceae and the genus *Juglans*. The natural range of *J. regia* extends from China in the east to Turkey in the west, and from Kazakhstan in the north to temperate regions of India and Nepal in the south. *J. regia* is grown commercially in China, USA, France, India, Italy and Spain for nut production. Juglone ($C_{10}H_6O_3$; 1,4-napthaquinone, 5-hydroxy-8CI) is an aromatic compound found in all parts of the *J. regia* plants[2]. This chemical is one of the oldest known allelopathic compounds in the history of cultivation and is also used as an active ingredient in herbal remedies and commercial dye[2,3]. Juglone has been reported to inhibit the growth of oral bacteria and to be nematicidal to root-knot nematodes[4,5]. It is under study for its reported anti-carcinogenic effects[6,7].

Provenances are geographical origin of propagules and in life sciences their testing is used to assess the genetic basis of geographic variability and find the most suitable geographical races for particular situations[8,9]. Phytochemicals are being used as a tool for studying the pattern of genetic variation in plants, including tree species[10,11]. Juglone is a major phytochemical present in all parts of *J. regia*, and can be a potential genetic marker to study variation. The detection method of juglone from fresh leaves of *J. regia* by high performance liquid chromatography (HPLC) has been already reported[12]; it takes ten minutes to process each sample. This method may be time-consuming for assessing a large number of samples. Alternatively, micro plate reader could process 96 samples in ten seconds at a fixed monochromatic wavelength and hence is faster method. There have been reports of seasonal variation in juglone level in soil beneath *J. nigra*[13], but no reports regarding the genetic variation of this compound. The present study was conducted to understand variation in juglone content in the leaves of *J. regia* in the provenance trial of this species from 18 geographical origins at Little Wittenham (lat. 51.63°N; long. 1.20°E; altitude 50 m), Oxfordshire, UK.

Each geographical origin would be called as provenance in this paper (Table 1). The trial was laid in randomized complete block design with non-contiguous, multiple-tree plots within each block. Provenances were randomly located within each block, with each block comprising 100 trees. There are 14 blocks and planting was 5 m × 5 m. Trial was surrounded with one guard row of *J. regia* to reduce edge effects. Out of 18 provenances, only 12 (K2, K3, K4, K5, K6, K7, K8, K9, K10, K11, J1 and E1) had 25 or more individuals per provenance. Hence only these 12 provenances were considered for this study (Table 1). Leaf samples were collected from the trial during the first week of August 2004. One leaflet from the fifth leaf of the third branch from the tip of the leader (main shoot) of each live tree was collected. Samples were placed in labelled, sealable, polythene bags. These bags were immediately placed in a thermal insulated icebox with dry ice and subsequently stored in a deep freeze (–70°C).

Samples were prepared according to the method described by Gîrzu *et al.*[12]. To estimate juglone content per 100 g of dry weight of leaves, the following method was used. First, sub-sample was taken from the middle of each leaflet and its fresh weight was measured to four decimal places in grams. The sub-sample was wrapped in a paper towel and put in an oven for 72 h at 70°C. After cooling, the dry weight of the sub-sample was recorded, again to four decimal places in grams. The moisture content (mc) of a leaflet was estimated as

$$\mathrm{mc} = (w_1 - w_2)/w_1, \quad (1)$$

where w_1 and w_2 are the fresh and dry weights of the first sub-sample. From the remaining portion of the leaflet, the second sub-sample was taken and its fresh weight recorded. This sub-sample was macerated in 25 ml of chloroform for 1 h at room temperature in a fume cabinet (ASTEC model Monair). The chloroform solution was filtered (using Whatman No. 1 paper) and evaporated in a rotary evaporator (Buch Rotavapor R-114) in a hot-water bath (Buch B-480) at 50°C

Table 1. Geographical origin of provenances of *Juglans regia* in provenance trials at Little Wittenham, Oxfordshire, UK

Provenance						
Code	Name	Country	Latitude (°N)	Longitude (°E)	Altitude (m)	Origin
K2	Ak Terek	Kyrgyzstan	41.25	72.83	1390	Autoch.
K3	Ak Terek	Kyrgyzstan	41.29	72.82	1860	Autoch.
K4	Sharat	Kyrgyzstan	41.27	72.85	1620	Autoch.
K5	Yaradar	Kyrgyzstan	41.32	72.98	1260	Autoch.
K6	Shidan	Kyrgyzstan	41.27	72.79	1590	Autoch.
K7	Kyzyl Ungur	Kyrgyzstan	41.38	73.09	1400	Autoch.
K8	Katar Yangak	Kyrgyzstan	41.30	72.84	1900	Autoch.
K9	Kyok Saau	Kyrgyzstan	41.30	72.88	1830	Autoch.
K10	Kyr Sai	Kyrgyzstan	41.84	71.95	1320	Autoch.
K11	Tera kolt	Kyrgyzstan	41.82	71.94	1440	Autoch.
E1	Catalonia	Spain	42.00	3.00	175	Unknown
J1	Seed orchard	France	–	–	–	Tadjikistan

Autoch., Presumed to be autochtonous; –, Not available.

under low pressure for 5 min. The dry residue was recovered in 10 ml of HPLC-grade methanol and transferred to a 25 ml capped and labelled glass bottle.

The dry weight (w_2') of the remaining portion of the leaflet was estimated as

$$w_2' = w_1' - (w_1' \times \text{mc}), \quad (2)$$

where w_1' is the fresh weight of the remaining portion.

For fast estimation of juglone, a method using VERSA max™ tunable micro-plate reader (Molecular Devices Corporation) was applied. The micro-plate reader can analyse 96 samples (in the wells of a plate) in 10 s at a fixed monochromatic wavelength, and produce readings of absorbance in a range 0.050–3.000 units. In the case of juglone, the detection wavelength was known (420 nm). After extraction, juglone samples were immediately transferred to wells of the micro-plate using a 200 µl adjusted micropipette (Eppendorf) and run through the micro-plate reader. The results were in print format and were transferred to a MS-Excel file manually. The results were expressed in absorbance/200 µl, and thus had to be converted to mg of juglone per g dry weight of leaf. A calibration standard was prepared with juglone (97%, Sigma Aldrich). For this purpose, a stock solution was prepared by dissolving 30 mg of juglone in 50 ml of methanol (HPLC grade). Five concentrations (6 mg/10 ml, 3 mg/10 ml, 1.2 mg/10 ml, 0.6 mg/10 ml and 0.12 mg/10 ml) were prepared from the stock solution. Thereafter, to five wells of a micro-plate, 200 µl of each of the five concentration solutions was transferred using a micro-pipette. The micro-plate with samples was run through the micro-plate reader and the results are presented in Table 2. A calibration curve was developed from these results using MS-Excel (Figure 1).

The linear equation for the calibration was

$$y = 0.8107x - 0.0464, \quad (3)$$

where y is juglone (mg/10 ml methanol) and x the optical density absorbance. Juglone (mg/10 ml methanol) expressed in eq. (3) is equivalent to juglone (mg/green weight of sample). Using eqs (1) and (2), the green weight of each sample was converted to dry weight. For statistical analysis, juglone (mg/dry weight of sample) was further converted to juglone (mg/100 g dry weight of sample).

The GENSTAT 8 (VSN International) statistical package was used for all data analysis. All data satisfied the requirement for homogeneity of variances between provenances. The observations of juglone content for provenances were not normally distributed; so a Box Cox transform with a cubic root was used to produce transformed data that satisfied the requirements for normality. The ANOVA procedure was used where provenances and blocks were treated as random effects; testing for significance of differences between provenances and blocks was therefore not appropriate, and F values were not calculated.

The mathematical model used for analysing provenance data is as follows[14]:

$$X_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where $i = 1$ to 12 provenances and $j = 1$ to 14 blocks, X_{ij} is the individual observation in the ith provenance and jth block, μ the population mean, α the provenance effects, β the block effects and ε_{ij} the residual term.

Using micro-plate reader juglone content of 96 samples was assessed in 10 s and is quicker than the HPLC method which takes 10 min to analyse one sample of juglone[12]. Variance component (%) on the basis of analysis of variation for 7-year height, 7-year collar diameter and juglone content is shown in Table 3. For juglone content, 1.22% of the total variation was explained by differences between provenances and 77.96% by differences between trees within provenances (residual term in Table 3). Variance component due to differences between provenances for juglone content was only 1.22 (%), which is less compared to

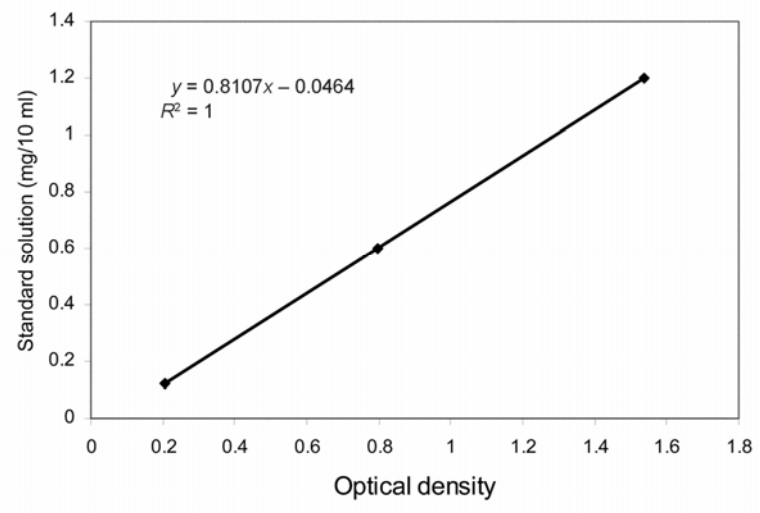

Figure 1. Calibration curve for juglone.

Table 2. Absorbance of five concentrations of juglone (mg/10 ml methanol)

Juglone (mg/10 ml)	Optical density (absorbance)
6.0	*
3.0	*
1.2	1.538
0.6	0.796
0.12	0.206

*Micro-plate reader could not read optical density more than 3.

Table 3. Variance component (%) for 7-year height, 7-year collar diameter and juglone content on the basis of analysis of variation in a provenance trial of *J. regia* in Oxfordshire

Source	Variation component of height (%)	Collar diameter variation (%)	Juglone content variation (%)
Provenance	14.16	14.54	1.22
Block	4.88	2.44	11.32
Provenance × block	17.62	15.77	9.50
Residual	63.34	67.94	77.96

Table 4. Mean juglone content (mg/100 g dry weight) of 12 *J. regia* provenances growing in a trial in Oxfordshire

Provenance code	Mean juglone content (mg/100 g dry weight)	Minimum	Maximum
K2	393.2	97.7	928.0
K3	369.1	21.5	1556.0
K4	313.8	13.1	1082.0
K5	339.7	38.5	1008.0
K6	362.3	37.0	1036.0
K7	384.3	44.4	1247.0
K8	393.3	33.1	1169.0
K9	347.7	27.3	1326.0
K10	348.0	25.0	1364.0
K11	371.6	23.5	1471.0
E1	295.3	20.0	634.0
J1	304.1	57.6	690.0
Overall	357.0	13.1	1556.0

7-year height (14.16%) and 7-year collar diameter (14.54%).

The overall mean juglone content in the trial was 357.0 mg/100 g dry weight (Table 4). The minimum value of juglone content was 13.1 mg/100 g dry weight and maximum value was 1556.0 mg/100 g dry weight. The mean juglone content was greatest for provenance K8 followed by provenance K1 (both Kyrgyzstan) and least for provenance E1 (Spain; Table 4).

The mean juglone content (357.0 mg/100 g dry weight) found in this study is consistent with the value reported by Gîrzu *et al.*[12], though values ranged widely (13.1–1556.0 mg/100 g dry weight) between the 1121 leaf samples from 12 provenances (Table 4). In provenances from Kyrgyzstan (K2 to K11), within the natural range of *J. regia*, leaf juglone content varied from 13.1 to 1556.0 mg/100 g dry weight, which is wider than the range of juglone content 20.0–690.0 mg/100 g dry weight of provenances from the other regions (E1 and J1) where the species had been introduced. The reduced variation in juglone content in provenances from the regions of introduction may be the result of a directional selection for quality nut production, if juglone affects the taste and/or smell of the nuts. Variation in the juglone content of fruit kernel and pellicle was reported in 10 varieties of *J. regia* in Slovenia[15]. The range of juglone content was 7.27–19.16 mg/100 g dry weight in kernels and 190.47–727.48 mg/100 g dry weight in the pellicle. Two varieties (A-117 and Rasna) had significantly higher amounts of juglone[15].

According to the study variation between trees was greater than variation between provenances of *J. regia* for juglone content (Table 3). Hence, the material tested in this study could be selected and desirable individual trees (those with either high or low juglone content, depending on breeding objectives) cloned by vegetative propagation instead of provenance selection. This approach may be more useful for achieving a quick and short-term objective of either increasing juglone content for juglone production or decreasing its content for nut production in horticulture and reducing allelopathy in agroforestry. Juglone assessment by micro-plate reader is a quick method for a large number of samples.

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