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DNA fingerprinting and anti-inflammatory activity of *Ocotea bullata* bark from different locations

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***Ocotea bullata* was sampled from forests at Mhlahlane, Zimankulu, Saasveld and Bangeni Weza. The bark of this plant is frequently used as a fine powder to treat headache, urinary disorders and stomach problems. Old and young bark, twigs and coppice were tested for anti-inflammatory activity. The COX-1 inhibition of hexane extracts from the old bark samples showed low inhibition in this assay. There was little variation in activity between extracts from the different collection sites as well as between plants from the same region. The TLC fingerprints also revealed very little chemical variation between old bark material from the different locations as well as between plants from the same region. DNA was extracted from young leaves only as older ones rapidly turned brown during the extraction process. There were no genetic differences, within and across the different habitats, detected with the six RAPD primers that were selected for use.**

Introduction

Ocotea bullata (Burch.) Baill. (Lauraceae) is one of the most frequently used traditional medicinal plants in southern Africa. Cunningham (1988) reported that *O. bullata* is amongst the top 10 medicinal plants sold in traditional medicinal markets in southern Africa. The part mostly used is the mature bark (Mander 1997). Harvesting of medicinal plants was formerly an activity performed by traditional practitioners who only had a limited effect on plant resources (Cunningham 1988). Commercial harvesters are today the main suppliers of traditional medicine and this greatly affects the survival of medicinal plants (Cunningham 1988). The bark is ground to a fine powder and is taken as snuff or burned and inhaled to treat headaches, chest complaints, urinary complaints and stomach ailments (Hutchings *et al.* 1996, Zschocke *et al.* 2000a). The bark can also be drunk as a tea or applied as steam to treat pimples (Hutchings *et al*. 1996). Several compounds have been extracted from the bark: ocobullenone (Sehlapelo *et al*. 1993), its structural isomer, *iso*-ocobullenone (Drewes *et al.* 1995) and their precursor, $\Delta 8^1$-3,4,5-trimethoxy-3^1,6^1-dihydro-3^1,4^1-methylene-dioxy-6-oxo-8,3^1-neolignan, a neolignan ketone (Zschocke *et al*. 2000c). Owing to the conservation status of this plant, several conservation strategies have been suggested: a) plant part substitution (Zschocke *et al.* 2000b) whereby leaves and roots can be used instead of the bark; b) plant substitution (Zschocke and Van Staden 2000) whereby *Cryptocarya* species could substitute for *O. bullata*; and c) *in vitro* culture (Kowalski and Van Staden 2001). In order to preserve genetic diversity, it is necessary to determine the degree of genetic variation between and within *O. bullata* populations found in South Africa. RAPD analysis was chosen as the method of investigation due to its speed and technical simplicity (Williams *et al*. 1990), even though in terms of reproducibility and sensitivity other techniques may offer better results (Newbury and Ford-Lloyd 1993). RAPD has been employed extensively for different purposes; Rajaseger *et al*. (1999) used it to study genetic uniformity between and within *Ixora* populations and mutants, Rath *et al.* (1998) used it to study phylogenetic relationships among members of the Dipterocarpaceae family, and Nakai *et al.* (1996) to study geographic variation of plants. In this study, material from mature trees was collected from four different locations, and their chemical fingerprints and anti-inflammatory activity were determined. RAPD markers were used to obtain an indication of the degree of genetic diversity between different populations.

Materials and Methods

Plant material was sampled from Saasveld, Mhlahlane, Bangeni Weza and Zimankulu forests in March 2001 (Figure 1). Five trees were sampled per location and leaves, twigs, coppice and bark were collected as available. The collected plant material was carefully labelled, frozen in liquid nitrogen

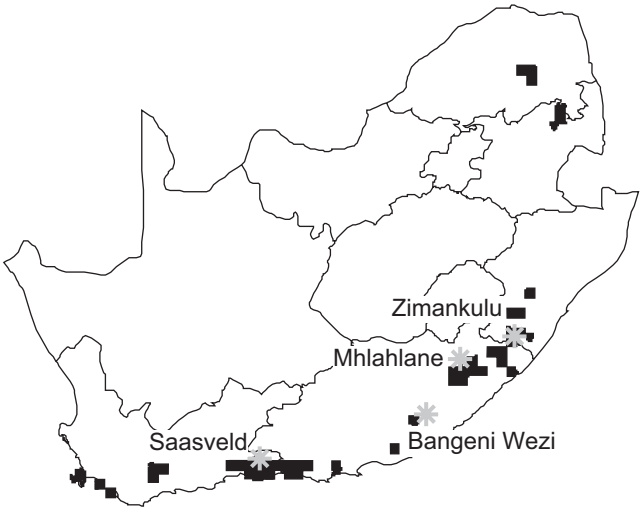

Figure 1: Sites from which *O. bullata* material was collected

and stored at –70°C for subsequent genomic DNA isolation and anti-inflammatory activity assay. Before storage all residual wood tissue was removed and the bark cleaned of lichens. Twigs were used whole.

Preparation of extracts and screening for anti-inflammatory activity

Bark samples, which had been stored at –70°C, were thawed, the materials dried at 50°C and then ground to a fine powder in a mill. Ground material (500mg) was extracted with 10ml hexane for 60min in an ultrasonic bath. The extract was subsequently filtered and taken to dryness. The extracts were redissolved in ethanol to a final concentration of 4mg ml^{-1} and assayed in duplicate for inhibition of COX-1 activity (Jäger *et al*. 1996).

TLC fingerprinting

Hexane extracts (500µl) were applied to Merck Silica gel 60 F_{254} Thin Layer Chromatography (TLC) plates (0.25mm) and developed using toluene:ethyl acetate 4:1 (v/v) as eluent. The plates were viewed under UV-light at 254nm and 366nm and under visible light after spraying with anisaldehyde-R (465ml ethanol, 5ml glacial acetic acid, 13ml concentrated sulphuric acid, 13ml p-anisaldehyde) and heating for 5min at 110°C.

Genomic DNA isolation

DNA was extracted from the leaves using the modified Hexadecyltrimethylammonium bromide (CTAB) extraction protocol (Richards 1997). Five hundred µl of CTAB extraction buffer (2% w/v CTAB, 100mM Tris-HCl, pH 8.0, 1.4M NaCl, 20mM EDTA, pH 8.0) were added to sterile 1.9ml Microfuge tubes already containing 3% (w/v) PVPP and allowed to re-hydrate for 20–30min in a 65°C water bath. Frozen leaf material (100mg) was ground to fine powders in a mortar and pestle using liquid nitrogen. Just before adding the ground leaf powder to the microfuge tubes, 1% (v/v) 2-mercaptoethanol was quickly added to the buffer in a fume hood. The contents in the microfuge tubes were mixed thoroughly and then incubated for 30min in a 65°C water bath. Thereafter, extraction with an equal volume of 24:1 (v/v) chloroform: *iso*-amyl alcohol was followed by centrifugation at 7 500g for 5min in a bench centrifuge. The aqueous phase was transferred into new microfuge tubes to which one-tenth the volume of CTAB/NaCl solution (10% w/v CTAB, 0.7M NaCl) was added. The contents were mixed well and the extraction with chloroform: *iso*-amyl alcohol was repeated. The aqueous phase was transferred into a new microfuge tube and an equal volume of CTAB precipitation solution (1% w/v CTAB; 50mM Tris-HCl, pH 8.0; 50mM EDTA, pH 8.0) was added. The mixtures were incubated in a 65°C water bath for 30min. Centrifugation (500g) for 5min in a bench centrifuge followed and the pellet was resuspended in 500µl high salt TE buffer (10mM Tris-HCl, pH 8.0; 0.1mM EDTA, pH 8.0; 1M NaCl). The DNA was precipitated by adding 0.6 volumes of ice-cold *iso*-propanol and pelleted at 7 500g for 15min. The supernatant was carefully removed and the pellet was washed once with 70% (v/v) ethanol and then with 100% ethanol. The pellet was air-dried to remove excess alcohol and thereafter dissolved in 20–50µl TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0). The DNA was quantified using a CARY 50 CONC UV-Visible Spectrophotometer and was diluted to 10ng μl^{-1} for RAPD analyses.

Random Amplified Polymorphic DNA (RAPD) analysis

The reaction mixture (25µl) for primer screening contained: 60ng template DNA, 1 x PCR buffer (Roche, Germany), 0.5µM 10-mer primer (Operon Technologies Inc., CA, USA), 0.2mM of each dNTP (Roche, Germany), 1.5 units of *Taq* DNA polymerase (Roche, Germany) and sterile HPLC grade water to make up to 25µl. The negative control mixture consisted of all reagents except the template DNA. Each reaction mixture was carefully overlaid with 30µl of mineral oil to prevent evaporation. The amplification was performed in an automated Hybaid Thermal Reactor (Hybaid Limited, UK) programmed for one cycle at 94°C for 1min, 36°C for 20sec and 72°C for 2min, followed by 45 cycles of 94°C for 10sec, 36°C for 20sec and 72°C for 2min, and the final extension cycle of 72°C for 5min and 35°C for 1min. From the primers selected at random, only four gave five or more reproducible bands following electrophoresis and were chosen for further analyses. Their sequences (5' to 3') were as follows: OPA-11: CAATCGCCGT; OPB-01: GTTTCGCTCC; OPB-04: GGACTGGAGT; and OPB-10: CTGCTGGGAC. The RAPD products together with a 100bp molecular weight marker were separated on 1.5% (w/v) agarose (Hispanagar, Burgos, Spain) gels, stained with 0.5µg μl^{-1} ethidium bromide and photographed using UVItec Gel Documentation System Doc-008 TFT. The bands were scored manually as discrete variables, using 1 to indicate presence and 0 for the absence of a band. Only intensely-stained bands were scored. The level of similarity as revealed by Single Linkage Hierarchical Cluster Analysis in Genstat™ 5 release 4.1 (1993) was determined.

Results and Discussion

When comparing the COX-1 inhibitory activity of old bark extracts from the different collection sites the activity was much the same at all sites, indicating that there were no major differences in anti-inflammatory activity between the populations (Figure 2).

Low percentage inhibition of COX-1 activity was detected in extracts from old bark; young bark was more active. Twigs and coppice gave the highest inhibition (Figure 2). The presence of chlorophyll compounds could be responsible for the high percentage COX-1 inhibitory activity detected in the younger plant materials and the higher inhibition may, therefore, be of little medicinal value. While the compounds responsible for COX-1 activity in *O. bullata* remain unidentified, it is possible to use twigs and coppice as substitutes for bark.

The TLC fingerprints showed little chemical variation amongst old bark from different trees and populations (Figure 3). The fingerprints from the different plant parts that were available from the same collection sites also showed similar chemical profiles for extracts after derivatisation with anisaldehyde-R and under 254nm UV-light (Figures 4A and B). However, under 366nm UV-light, pink bands were apparent in the lanes of young bark, twigs and coppice (Figure 4C). These bands are likely to be chlorophylls and/or breakdown products thereof.

The homogeneity in the populations is shown at the DNA level. Good quality of DNA was extracted from the young leaves while the older ones readily turned brown during the extraction process. No attempt was made to extract DNA from the barks, twigs and coppice as it is the old bark that is used medicinally. No DNA was extracted from Bangeni Weza as young bark and twigs were the only material collected from this location. The older leaves turned brown during the grinding in liquid nitrogen as a result of phenolic compounds that were released by the damaged leaves. The release of phenolic compounds was also reported in the *in vitro* culture of this species (Kowalski and Van Staden 2001). These compounds are released from the vacuoles and react with proteins and nucleic acids (Wang and Vodkin 1994). This negatively affects the DNA yield and purity. Their binding to the DNA was prevented by adding insoluble PVPP and 2-mercaptoethanol to the extraction buffer.

Results obtained with the six random primers used were homogenous across and within the three habitats investigated. This indicated that there was genetic uniformity within and across the three habitats. Only OPB-01 and OPB-04 primers were used to indicate the results (Figures 5A and B). The two primers yielded six and 13 bands respectively in the screening process. Their band sizes ranged from 3 530 to 564 base pairs in the former and from 3 530 to 947 base pairs in the latter. Primer OPB-01 with at least four monomorphic bands amplified a total of 125 bands. The similarity matrix classified all the plants into one group with a mean similarity of 72.8%. Primer OPB-04 yielded only one monomorphic band from a total of the 79 amplified bands. The similarity matrix also classified all the plants into one group with a mean similarity of 75.9%. The similarity in the

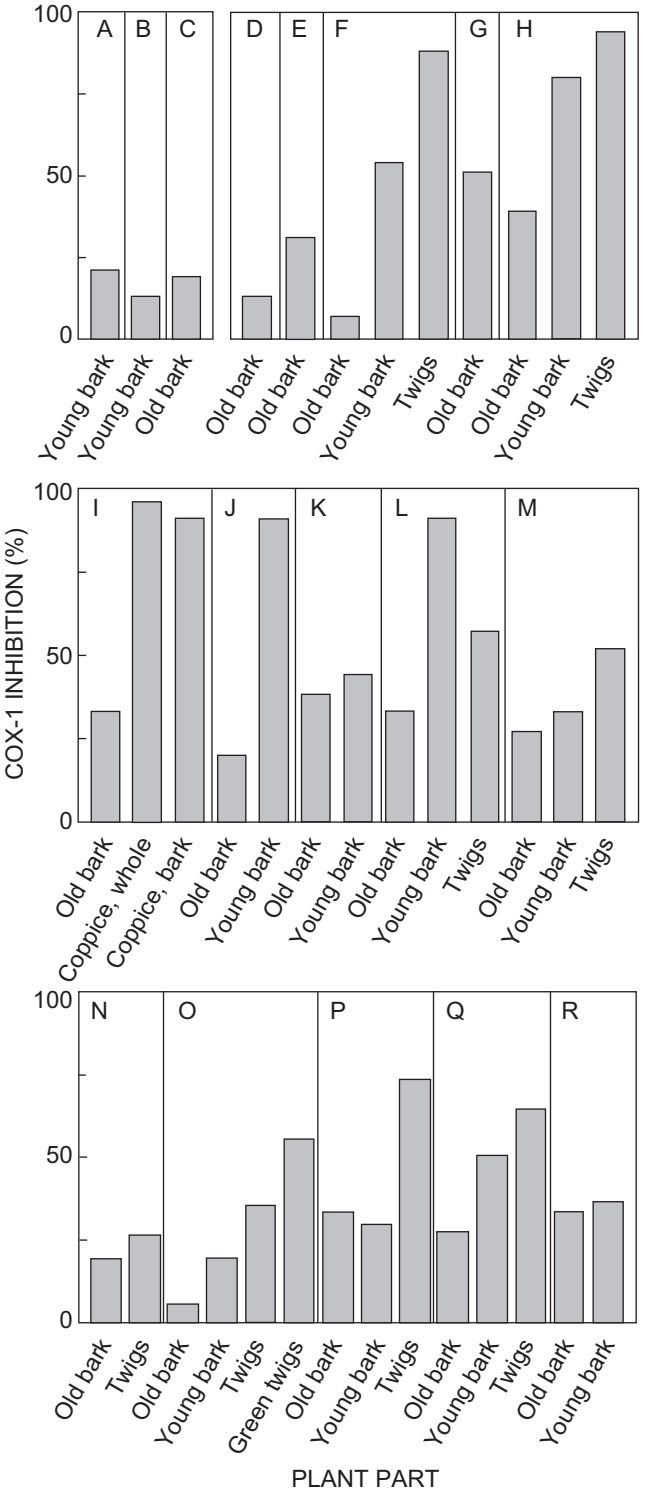

Figure 2: Inhibition of cyclooxygenase by extracts made from various plant parts of trees from different collection sites. Trees A–C = Bangeni Wezi; D–H = Zimankulu; I–M = Saasveld; N–R = Mhlahlane

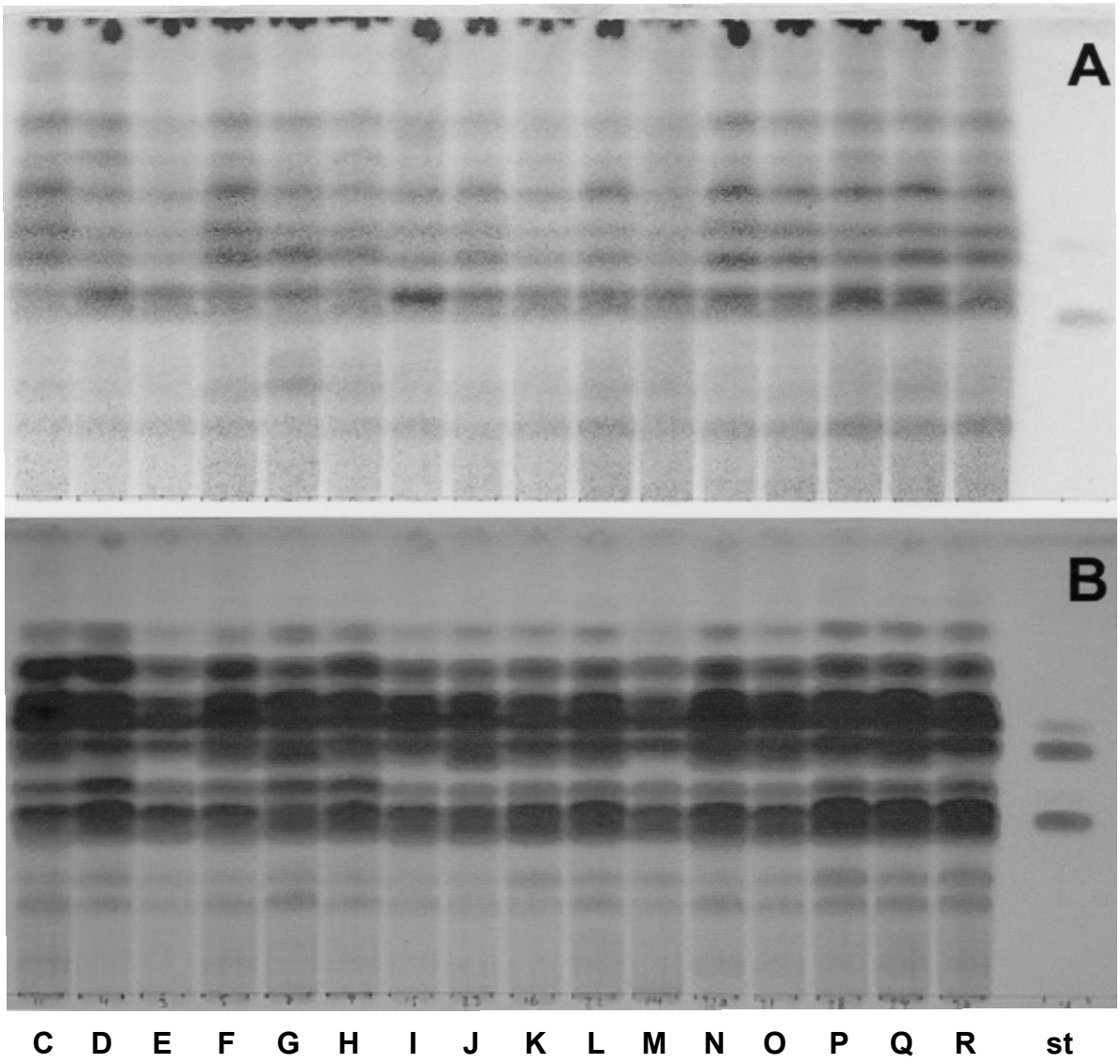

Figure 3: TLC fingerprints of extracts from old bark of trees from all collection sites: (A) VIS after derivatisation with anisaldehyde, (B) under 254nm UV-light. St: 'precursor' ($\Delta 8^1$-3,4,5-trimethoxy-3^1,6^1-dihydro-3^1,4^1-methylene-dioxy-6-oxo-8,3^1-noelognan), ocobullenone, *iso*-ocobullenone (order from origin), C = Bangeni Wezi, D–H = Zimankulu, I–M = Saasveld, N–R = Mhlahlane

genetic fingerprints supports the results found with chemical analysis for this plant. There is no record in the literature on the degree of genetic variation for this plant. The collection sites are very far from each other. However, it is possible that the results may not be accurately representing the whole species in each habitat due to a small sampling size. It is possible to also use twigs and coppice instead of bark as the chemical profiles and genetic fingerprints within and across habitats were very similar.

During the grinding of the plant materials it was evident that old bark was much easier to grind to a fine powder than the other plant parts. Twigs and young, fibrous bark were particularly difficult to grind. As sniffing of powder is the preferred way of administration, this could be a practical obstacle to use younger plant parts for substitution.

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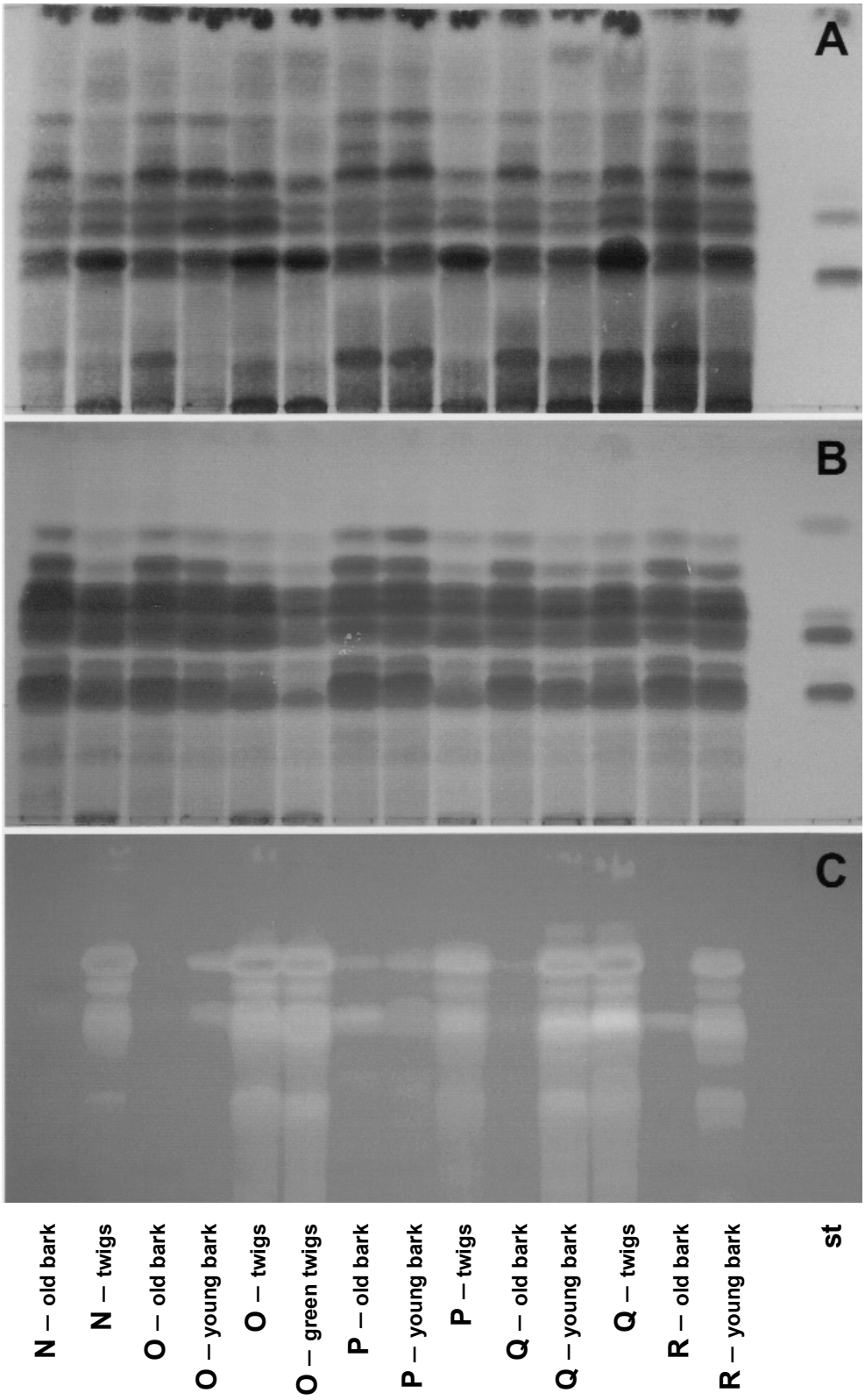

Figure 4: Extracts from various plant parts of trees N–R from Mhlahlane: (A) VIS after derivatisation with anisaldehyde; (B) under 254nm UV-light; (C) under 366nm UV-light. St: 'precursor' as for Figure 3

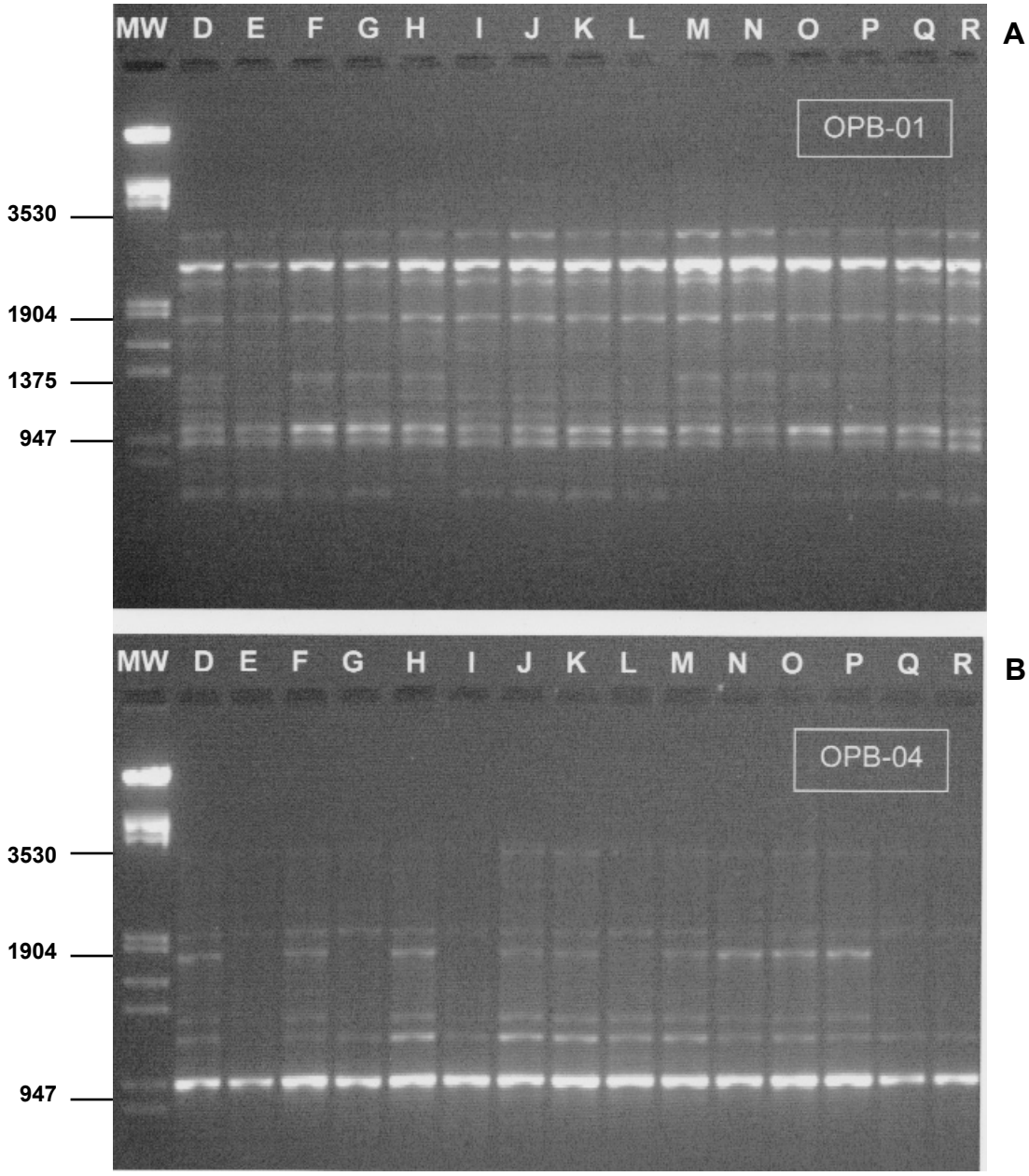

Figure 5: RAPD profiles of *O. bullata* collected from different locations generated with primers OPB-01 (A) and OPB-04 (B). Trees D–H = Zimankulu; I–M = Saasveld and N–R = Mhlahlane. Lane one represents the molecular weight marker III

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