GOMORTEGA KEULE: MICROPROPAGATION AND GERMPLASM CHARACTERIZATION

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Abstract

The endangered tree *Gomortega keule* (Mol.) Baillon belongs to the monotypic family Gomortegaceae and is restricted to a small area in Chile. It produces edible fruit and has other uses. However, there is no initiative aimed at its cultivation, presumably because of its difficult propagation. The literature was reviewed for *G. keule*. *In vitro* techniques were assessed as a basis for conservation and future domestication. Zygotic embryos, bud, stem, leaf and petiole explants were used in attempts to establish material *in vitro*. Cultures for shoot and root production involved evaluation of a range of plant growth regulators, concentrations, supplements and environmental conditions. The use of liquid media for tissue culture of *G. keule* and somatic embryogenesis was also explored. Contamination was the main hindrance to the establishment of cultures, particularly in explants derived from field-collected shoots. The optimum procedure to establish actively growing cultures of *G. keule* was the use of zygotic embryos as starting material on semi-solid WP medium with 0.1 mg/l NAA and 1.0 mg/l BAP. Stabilization of the material was a constraint, as tissues often showed hyperhydricity. The optimum conditions for proliferation of shoots of *G. keule* were on WP medium with 0.1 mg/l NAA, 1.0 mg/l BAP, 8 g/l agar, and 20 g/l sucrose at 18ºC. Phenotypically normal shoots developed on WP medium with 2 g/l activated charcoal. Rooting of those shoots was stimulated with a treatment of 1 week on medium with 20 mg/l IBA. Survival of the plants after acclimatization reached 65% after 8 months. Cultures of compact callus and small embryogenic aggregates showed optimum proliferation in liquid MS medium with 20 g/l sucrose, 0.01 mg/l NAA and 0.1 mg/l 2iP. Somatic embryogenesis was observed from compact calli and from small aggregates when they were transferred onto semi-solid MS medium with 0.01 mg/l NAA and 1.0 mg/l BAP. On the same medium, recurrent somatic embryogenesis was also observed directly from the radicle of zygotic embryos. Somatic embryogenesis was induced in shoots after 6.5 months on semi-solid MS medium supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l 2iP. Embryogenic callus proliferated after cryopreservation, which will complement long-term conservation efforts for *G. keule*. Chromosomes were observed in cells of embryogenic cultures, suggesting diploidy. Using 9 microsatellites, the genetic fidelity of cultured material and the genetic variation were assessed in natural populations of *G. keule*. Genetic variation was not detected in two embryogenic genotypes cultured *in vitro*. The populations of plants showed large genetic variation, indicating that conservation efforts must include southern populations.

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2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(γ,γ-Dimethylallylamino) purine
AC	Activated charcoal
ABA	Abscisic acid
BAP	6-Benzylaminopurine
BSA	Bovine serum albumin acetylated
CTAB	Hexadecyltrimethylammonium bromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GA_3	Gibberellic acid
HWE	Hardy-Weinberg equilibrium
HFS	High frequency subculture
IAA	3-Indole acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
LN	Liquid nitrogen
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthaleneacetic acid
PCO	Principal coordinate analysis
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PVP	Polyvinylpyrrolidone
SSR	Simple sequence repeat
TDZ	Thidiazuron
WP	Woody plant medium (Lloyd and McCown, 1981)
Ztn	Zeatin

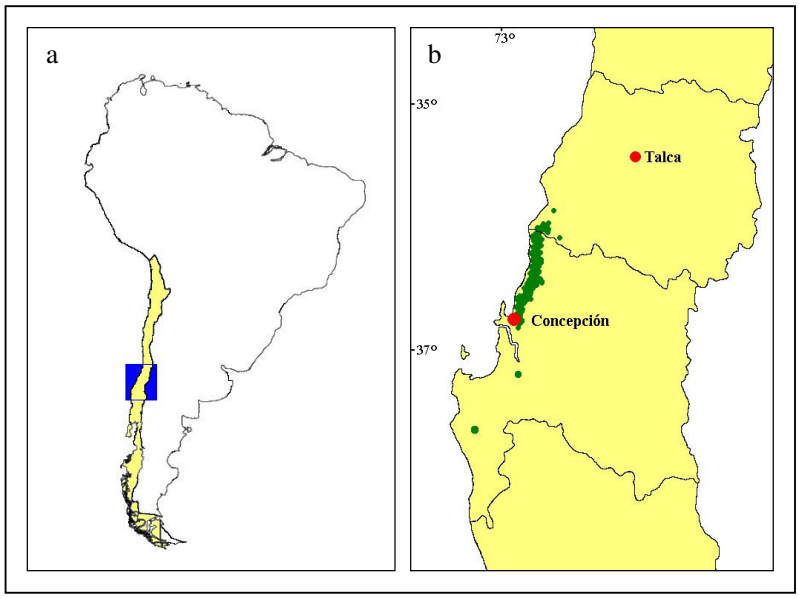
Chapter 1

General review of *Gomortega keule*

1.1 Introduction

The family Gomortegaceae has one genus with one species (Reiche 1934; Reiche 1937), namely the tree *Gomortega keule* (Mol.) Baillon, and is the only endemic and monotypic family in the Chilean continental flora (Marticorena 1992). *G. keule* is restricted to a geographical area of the central coast of Chile (Figure 1.1), where it is endangered and in drastic need of conservation. It produces edible fruit and has several other uses, particularly as a source of timber. Surprisingly, the tree has never been cultivated. *G. keule* is of considerable botanical interest because it represents an archaic angiosperm genus (Zizka 1992). However, there are large gaps in knowledge relating to this genus, Hutchinson (1964) referring to it as a "little-known and puzzle tree". The literature relating to *G. keule* is dispersed; some represents limited research or information from diverse sources, but which is of value in its own right. Several of the published reports such as those of Becker (1964), Chaudefaud and Emberger (1960), Font Quer (1964), Hernández (1970) and Mabberley (1997), discussed *G. keule* but did not cite the background references. Other citations are incomplete, as in the publications of Murillo (1861), Moesbach (1944, 1992), Hedrick (1972) and Hoffmann *et al.* (2001). Some reports repeat previous findings with little new information, although images have been added to the text in a few cases (Muñoz 1959, 1966; Hutchinson 1964, Muñoz 1966, González *et al*. 1991; Hoffmann 1997; Sampson 2000). This chapter summarizes many publications that are dispersed throughout the literature, provides an overview of *G. keule*, and presents a case for its conservation.

Figure 1.1. Natural geographic distribution of *Gomortega keule*.



a) Chile. The area in blue is inlarged in b).

b) VIIth and VIIIth administrative regions of Chile, showing capital cities and *G. keule* populations.

1.2 Taxonomy of *Gomortega keule*

The first European document relating to the taxonomic information on *G. keule* was composed by Molina (1782), who gave the tree the name *Lucuma keule*. Later, Ruiz and Pavon (1794) placed the tree in the genus *Gomortega*, creating this name as a truncated fusion of Gomez Ortega, who supported their expedition to South America from 1777 to 1788. The genus name was maintained in a later publication by the same authors (Ruiz and Pavon 1802). A second scientific name, *G. nitida*, was proposed by Ruiz and Pavon (1798), followed by *Adenostemum nitidum* (Persoon 1805). Gay (1849) and De Candolle (1864) also described the species under this last name. Molina (1810) changed the name to *Keulia chilensis*. Baillon (1869) referred to the tree as *G. keale*, a typographic error as indicated by Espinosa (1948). Mez (1889) and Engler and Prantl (1900) used the name *Gomortega nitida* Ruiz et Pav. and, finally, Johnston (1924) proposed the name *G. keule*. The latter scientific name, *Gomortega keule* (Mol.) Baillon, presently in use, was clarified by Espinosa (1948) and confirmed by Rodríguez *et al*. (1983). Some authors (Donoso 1984; Heusser 1971; Heo *et al*. 2004; Renner 1999; Renner *et al*. 2000) have used the name *G. keule* (Mol.) Johnston, or even *G. nitida* Ruiz et Pav., although such names are considered invalid.

The oldest explicit reference to the species in terms of its common name was given in the original work of the Jesuit Father Diego de Rosales written in 1674, where he referred to the tree as *queul* (De Rosales, 1877), this name also being mentioned by Febrès (1765) and Valenzuela (1919). This would be the archaic Mapuche name according to Gunckel (1959), changing then to *queuli*, as mentioned by Havestadt (1777), Lenz (1910), Valenzuela (1919) and Moesbach (1992), or its variant *keuli*, and finally to *queule*. Although Molina (1782) did not indicate a common name for the species, he gave the specific epithet *keule*. Bullock (1932) emphasised that the abbot Molina attempted to preserve the Araucanian (Mapuche) names in the scientific names that he created. In fact, *keule* is pronounced the same as *queule*, the common name indicated by Ruiz and Pavon (1797). These authors again cited both names, *keule* and *queule*, in a later publication (Ruiz and Pavon 1798). Gunckel (1966) mentioned *keulia*, used by Molina (1810), as a Spanish version of the name. Gay (1849) reported the common names *queule* and *hual-hual*. Valenzuela (1918) stated the tree was called *hualhual* and *hualhualn*, meaning the sound and wobbling produced by a moving liquid. According to Gunckel (1959), the last name is an old Mapuche name that is now out of use. Havestadt (1777) mentioned the word *hualhualun* with a similar meaning, as for liquid in the stomach, or a gargle. However, Moesbach (1992) stated *hualhual* means girth, referring to the rounded compact shape of the crown of the tree. Santa Cruz (1932) indicated that the name *queule* was also used to designate the fruits, while other authors (Baeza 1930; De Candolle 1864) referred to the names mentioned earlier.

Queule also appears in the Chilean toponymy as the name of a river and a small fishing harbour, both at latitude 39º 23' South. Lenz (1910) stated that the name of three farms in Itata, Concepción and Coelemu included the name *queule*, while more recently, Peña (1995) used *G. keule* material obtained from a forestry farm with the former name, near Concepción. Interestingly, Ruiz stated that, according to natives, there was a different species of *queule* growing in the south of Chile from Arauco to Valdivia, with smaller, pointed seeds (Jaramillo-Arango 1952; Ruiz 1998), but this species has never been found or described.

1.3 Morphology of *Gomortega keule*

Several reports have mentioned different heights and diameter of the trunks of trees (Figure 1.2a). Specimens have been described up to 15 (Rodríguez *et al*. 1983; San Martín and Donoso 1996; Del Fierro 1998), 20 (Albert 1924), 25 (Muñoz 1962; Donoso 1984; Cabello

1987b) and 28 m (Dohlen 1999) in height, with a mean trunk diameter of 15 (San Martín 1997), 30 (San Martín and Donoso 1996), up to 60 (Rodríguez *et al*. 1983), 80 (Donoso 1984), 90 (Dohlen 1999) or 100 cm (Albert 1924). One tree, 110 cm in diameter, was observed by Villegas *et al*. (2003), while Espinosa (1948) observed the base 180 cm in diameter of the trunk of an old, logged tree. Cabello (1987b) reported the tree has a monopodic habit of growth, but this phenotype is more typical of young trees, as old trees are sympodic.

The tree bears evergreen leaves (Cronquist 1981; Kubitzki 1993) that are opposite, lack stipules (Baillon 1869; Kubitzki 1993), are 5.0 - 11.5 cm in length and 2.0 - 4.5 cm wide (Espinosa 1948; Rodríguez *et al*. 1983), with a petiole 0.8 - 1.5 cm in length. The young stems are quadrangular in transverse section (Kubitzki 1993; Hechenleitner *et al*. 2005). Gil (1932) described the adaxial face of the leaf as having a thick cuticle and an epidermis with a double-layered hypodermis as confirmed by Rodríguez (2004); the palisade parenchyma has essence-bearing cells, the latter also being found in the spongy parenchyma. Rodríguez (2007) measured the density of idioblasts in the leaf layers and observed them in the adaxial epidermis (density 1.85/mm^2) and the mesophyll. Rodríguez (2004) also reported idioblasts of average diameter 67 μm which were distributed randomly in the tissue. In addition, Cronquist (1981) reported spherical cells with essential oils in leaves and young stems, with small needles or prisms of calcium oxalate in some cells. Similarly, Kubitzki (1993) described a multi-layered epidermis and secretory cavities in parenchymatous tissue of the stem and leaves. The foliar architecture (Barrera 1992) and foliar epidermal characteristics (Barrera and Meza 1992) of *G. keule* have been described in detail, as a basis for comparative studies in paleobotany and systematics.

Figure 1.2. Photographs of *Gomortega keule* in its natural habitat.

a) Young trees in Ralbún, VIIth region.

b) Flowers. Scale bar = 5 mm.

c) A seedling in Ralbún.

d) Fruits, the halved one showing the endocarp.

e) An open endocarp showing a seed. Scale bar = 15 mm.

f) A zygotic embryo. Scale bar = 3 mm.

g) Shoots circularly arranged around an old rotten stump.

The flowers of the tree are hermaphrodite (Figure 1.2b), gathered in terminal racemes on short opposite pedicels (Baillon 1869), 6 - 14 in number, and point downwards (Espinosa 1948). However, they are unattractive and green-creamish in colour (Serra *et al*. 1986) with a diameter of 5 - 7 mm (Rodríguez *et al*. 1983). Del Fierro (1998) mentioned the flowers to be very aromatic, a fact not stated elsewhere. From their genetic study, García-Gonzáles *et al*. (2008) suggested *G. keule* has an allogamous breeding system. Several authors have discussed floral morphology (Gay 1849; Philippi 1865; Baillon 1869; Mez 1889; Reiche 1896; Engler and Prantl 1900; Stern 1955; Buchheim 1958, 1959). Brizicky (1959) reviewed this subject and concluded that the tepals (3.0 - 3.5 mm in length) are 5 - 9, but often 7, spirally arranged and becoming smaller towards the inside. Stamens (1.5 - 3.5 mm in length) are 7 - 13, also spirally arranged and also becoming smaller towards the inside. One to 3 exterior stamens are tepal-like, bearing 2 anthers at the top and sometimes 1 or 2 glands at their bases. The other 5 - 10 stamens, often 8, are typical in structure with a filament and valvate anther, usually bearing at the base one stipitate, ovate to reniform gland on each side. Around the style there are 1 - 4, usually 3, staminodia each 1.0 - 1.5 mm in length. The ovary is inferior, with 2 - 3 locules; the style has 2 or 3 stigmatic branches (Brizicky 1959). The 2, or rarely 3, carpels each have a single ovule which aborts easily (Baillon 1869). Consequently, 1 seed is normally found in an endocarp. Leinfellner (1968) observed 2 - 3 spirally arranged carpels, although Doweld (2001) reported 3 - 6 similar structures with the basal ones often being abortive, confirming the spiral arrangement. Endress and Igersheim (1997) noted the carpel wall to be highly tanniferous with cells containing oil, druses and crystals. The pollen of *G. keule* is spherical and rugged, as in other members of the Lauraceae (Mez 1889), or granular and 25 - 31 μm in diameter (Erdtman 1952). Heusser (1971) reported the pollen to be monad, apolar, inaperturate and spheroidal, 34 - 50 μm in diameter. The sporoderm structure was studied by Hesse and Kubitzki (1983), who confirmed pollen to be inaperturate, with a thin exine and a thick, bi-layered intine.

The fruit of *G. keule* is a false drupe that develops from an inferior ovary, yellow in colour when ripe (Figure 1.2d; Ruiz and Pavon 1798; Hechenleitner *et al*. 2005), 3.5 - 7.0 cm in length and 2.0 - 5.0 cm in diameter (Rodríguez *et al*. 1983; Le Quesne and Stark 2006). Serra *et al*. (1986) recorded a fruit weight of 10 - 24 g while Muñoz *et al*. (2003) reported an average of 28.2 g, with 71 - 78% of the fruit weight (17.5 g) corresponding to the pulp (Le Quesne and Stark 2006). Cells with essential oils are present in the mesocarp (Doweld 2001). The lignified endocarp, a putamen, is a very hard nut, stony or woody, 21 - 24 mm in length, 17 - 19 mm in diameter and 3.7 - 6.8 g in weight (Figure 1.2e; Donoso and Cabello 1978; Serra *et al*. 1986; Aburto 2002). Le Quesne and Stark (2006) observed variation in the morphology of the endocarp and reported that 76% of endocarps bore 1 seed, 23% had 2 seeds

while 1% had 3 seeds, the latter being flattened, 10 - 14 mm in length, 5 - 10 mm wide, 4 - 6 mm thick and 90 - 125 mg in weight (Rodríguez *et al*. 1983; Doweld 2001; Aburto 2002; Le Quesne and Stark 2006). The seed is albuminous (Chaudefaud and Emberger 1960; Doweld 2001) with a copious endosperm that contains oil droplets; the embryo is straight and symmetrical (Figure 1.2f; Heo *et al*. 2004). There is discrepancy relating to embryo size, considered small by Baillon (1869) and Doweld (2001), or rather large by others (Chaudefaud and Emberger 1960; Cronquist 1981; Kubitzki 1993). Le Quesne and Stark (2006) reported some seedlings had 3 cotyledons.

1.4 Systematics

De Candolle (1864) described the species in the family Lauraceae, while Philippi (1865) suggested its position between the Lauraceae and Monimiaceae, as also noted by Chaudefaud and Emberger (1960). Baillon (1869) placed *G. keule* in the family Monimiaceae, creating a new tribe within the family for this monotypic taxon. However, he stated its affinity with the family Lauraceae. Reiche (1896) created the family Gomortegaceae with its genus and sole species, as it is at present. *G. keule* has affinities with members of the Lauraceae and Monimiaceae based on xylem anatomy, which shows primitive characteristics (Stern 1955). Cronquist (1981) allied the species to the family Monimiaceae, in agreement with Takhtajan (1980), who reported the family to be closely related to the Monimiaceae, and within the latter, especially to the group considered to be the distinct family Atherospermataceae. Hesse and Kubitzki (1983) studied the sporoderm, and reported that *G. keule* may represent an intermediate stage in the evolution of the Laurales, with a unique and peculiar exine structure within the angiosperms, although sharing some characteristics with the Lauraceae and Hernandiaceae. In a comparative study of the gynoecium of the Laurales, Endress and Igersheim (1997) confirmed that the families Lauraceae, Hernandiaceae and Gomortegaceae, share important characteristics and form the core group of the order. Using morphological comparisons and analysing chloroplast DNA sequences, Renner *et al*. (1997, 2000) stated that the closest relative to the Gomortegaceae is the Atherospermataceae, the closest relative to both of these families being the Siparunaceae. Doweld (2001) studied seed and fruit characteristics, and concluded the family has phylogenetic affinities with the Monimiaceae and, especially, with the Atherospermataceae. Studies on the embryology of the Laurales (Kimoto and Tobe 2001, 2003) revealed some synapomorphic characters, and thus close affinities, in the Siparunaceae, Gomortegaceae and Atherospermataceae, in accordance with the phylogenies proposed by Renner (1998, 1999, 2002). Eklund (2000) discussed stamen

evolution in the order and also agreed with the work of the last cited author. In a detailed histological investigation, Heo *et al*. (2004) observed the embryology of the Gomortegaceae and its relation within the Laurales, especially with the Atherospermataceae and Siparunaceae, although they recommended additional investigations to clarify the relationship.

Cytologically, Goldblatt (1976) recorded a chromosome number of $2n = 2x = 42$ ($x = 21$) and indicated a palaeohexaploid condition, again suggesting a close affinity with the families Atherospermataceae and Siparunaceae. More recently, Baeza *et al*. (2001) confirmed the same number of extremely small chromosomes, each less than 3 μm in length. Oginuma and Tobe (2006) showed an archaic base number of $x = 11$ within the Laurales and suggested polyploidization from $x = 11$ to $x = 22$ in the Gomortegaceae, with an aneuploid decrease from $2n = 44$. These authors emphasised the need for confirmation of the cytology of *G. keule*, since 42 chromosomes represent a sufficiently large number to miscount.

G. keule may be a remnant from ancient floras of the Eocene epoch (Serra *et al*. 1986). However, the only fossil record for the family is from the late Oligocene to early Miocene deposits in Chilean Patagonia (Nishida *et al*. 1989). Renner (2005) suggested an even older origin dating back 100 million years, inferred from molecular data. *G. keule* has been listed amongst several endemic, generally monotypic taxa with a discontinuous distribution, that are restricted to the shore and the west slopes of the coastal mountain range, the Cordillera de la Costa (Armesto *et al*. 1996; Villagrán *et al*. 1998; Villagrán and Armesto 2005). Probably, most of these species are ancient endemic species which had a more extensive distribution before and after the glacial periods. These plants may have persisted because of the oceanic climate which allowed, in that area, the existence of a refuge (Villagrán *et al*. 1998). Hinojosa *et al*. (2006) reported that the mixed palaeoflora, which developed in Central Chile during the Oligocene to Miocene transition (*ca.* 25 million years ago), is closely related to the remnant deciduous Maulino forest. In presenting a contrasting view, Renner (2005) discussed the limited number of species for some groups within the Laurales and considered the hypothesis that ancient lineages, like *G. keule*, persisted in habitats with limited biological competition, such as terrestrial islands and, because of their close local adaptation, they could not extend into other habitats.

1.5 Conventional propagation

There is general agreement in the literature that the tree flowers and fruits mature in the autumn (Table 1.1). According to Espinosa (1948), new flowers are produced while fruits ripen. San Martín (2005) reported that most flowers are lost because of rain, winds and low temperatures, the same author noting that fruit maturation is completed when flowering commences the following season. Rodríguez (1988) and Riedemann and Aldunate (2003) also reported that the fruits mature during autumn of the next year, suggesting that about 12 months are required for fruit maturation. Similarly, Le Quesne and Stark (2006) observed that flowers and fruits in different developmental stages may occur on the same branch. Consequently, more than one season may be required to complete fruit maturation. This agrees with the observation of Ruiz and Pavon (1798) that fruits and flowers can be found throughout the year. After fruit maturation and fall, the flesh rots when the ripe fruits are on the forest floor, freeing the endocarp (Muñoz 1986; San Martín and Donoso 1996).

Table 1.1. Times of flowering and fruit production in *Gomortega keule*.

Stage	Time	Reference
Flowers	February to June	San Martín (2005)
Flowers	March	Ruiz and Pavon (1798)
Flowers	March to April	Rodríguez (1988); Riedemann and Aldunate (2003)
Flowers	March to June	San Martín (1997)
Flowers	April to May	Cabello (1987b)
Flowers	End of April onwards	Rodríguez *et al*. (1983)
Flowers opening	Beginning of May	Espinosa (1948)
Flowers	All year	Ruiz and Pavon (1798); Ruiz (1998)
Fruit maturation	February to May	Cabello (1987b); San Martín (2005)
Fruit maturation	March to May	Muñoz (1986); Rodríguez (1988); Riedemann and Aldunate (2003)
Fruit maturation	April to May	Donoso and Cabello (1978)
Fruit maturation	End of April	Donoso (1984, 1995)
Ripe fruits	Late March to early May	Rodríguez *et al*. (1983)
Fruit fall	March to June	San Martín (1997)

Fruits may be harvested with long poles, by climbing the trees, or collecting from the forest floor (Cabello 1987a). The seed is difficult to extract mechanically from the endocarp and is

easily damaged (Le Quesne and Stark 2006). The humidity of the endocarp is normally 11.3 - 36.7% (Serra *et al*. 1986), this being 13% after dry storage, 20% after soaking in water for 2 days, with a seed humidity of 5 and 30% after dry storage and 5 days of soaking, respectively (Orellana 1996).

The seed undergoes epigeal germination (Donoso 1984). In a subsequent publication, Donoso and Escobar (1985) reported 24% germination in the spring, 5 months after collecting seeds from the forest floor, and stated that seeds do not germinate in the same autumn as they are harvested. In agreement, Cabello (1987b) and Serra *et al*. (1986) indicated that seeds collected in the autumn and planted immediately germinated mainly in the following spring, while seeds collected from the forest floor and planted in winter began to germinate 4 - 5 months later. Maldonado (1990) reported only 4% germination after 11 months; Dohlen (1999) also observed very low germination rates. Seeds did not germinate following chemical scarification with sulphuric acid (Rodríguez 1988). Orellana (1996) studied seeds collected from the forest floor the previous season; when the seeds were treated with a solution of 1% (w/v) gibberellic acid (GA_3) for more than 48 hours, germination commenced after 110 days, reaching 17% after 5 months. In contrast, germination was not observed in untreated seeds. Using seeds that were also naturally scarified on the forest floor, Espejo *et al*. (2000) reported 21% radicle emergence after 3 weeks following treatment of seeds with 1000 ppm GA_3. Morales and Calquin (2004) collected fruits in April and May, scarified the endocarps mechanically with a hammer and chisel, and placed the seeds in compost. Although germination commenced after 5 months, it was not more than 2% after 14 months, but 42% after 18 months, *i.e.* in the second spring after seed production. The long period required for germination in these experiments may reflect the requirement for embryo maturation and degradation of the endocarp (Maldonado 1990). The latter author observed loss of viability with seed ageing. Aburto (2002) and Jara (2006) failed to germinate seeds extracted from endocarps because of contamination by microorganisms, and suggested the endophytic origin of the microorganisms. Muñoz *et al*. (2005) attributed seed contamination to fungi and bacteria and recommended that seeds should not be extracted from the endocarps for propagation purposes. Seedlings of *G. keule* grow slowly in the first season (Serra *et al*. 1986) reaching a height of only 5 cm in one year (Cabello 1987b).

One of the few references to the growth of *G. keule* in the field (Rodríguez 1988) also emphasised that the trees grow slowly, reaching only 25 - 40 cm in height in 3 years. Subsequent growth is more rapid, plants being 2 - 3 m in height within 10 years. Riedemann and Aldunate (2003) also mentioned that the tree initially grows very slowly (approx. 10 cm/year) increasing in the fourth year and reaching 1 m in 6 years. More accurate data were

provided by Navarrete (2006), who reported an annual mean growth in height of 26 cm for the first year, increasing to 48 cm in the 18th year, the mean trunk diameter stabilising to about 1 cm per year after 17 years. The same author discussed the poor growth in cultivation compared to that of trees in their natural habitat, with some problems being manifest mainly in the foliage, possibly induced by poor soil with low concentrations of phosphorus and potassium, but high concentrations of aluminium.

Rooting of cuttings was extremely difficult (Peña 1995), with only 3.3% of cuttings developing roots after 5 months when collected in October and dipped for 300 sec in a solution of indolebutyric acid (IBA) of 6 g/l. Callus formation occurred at the base of 11.6% of the cuttings collected in September. The incubation temperature of 26ºC may have had a negative role in these experiments. In later work, Peña *et al*. (1996) suggested that the phenolic content of shoots did not influence rooting, despite the high concentration of these compounds in *G. keule* compared to other trees. More recently, Hechenleitner *et al*. (2005) reported that it was possible to root 10% of cuttings following treatment with growth regulators, although shoot growth was often plagiotropic in the resulting plants. Despite these problems, Le Quesne and Stark (2006) maintained that it was possible to obtain plants with good development and roots from cuttings.

1.6 Micropropagation

Micropropagation may provide an additional approach to the multiplication of *G. keule*. Indeed, some effort has been directed to propagation of this tree *in vitro*. For example, Barrales *et al*. (1983) pioneered a tissue culture strategy, inducing growth from lateral buds collected in May. This approach was continued by Baeza *et al*. (1986) using apical and lateral buds on culture medium of unspecified composition supplemented with 1.5 mg/l benzylaminopurine (BAP), 1.0 mg/l GA_3 and 0.1 mg/l naphthaleneacetic acid (NAA). Buds opened after 15 days and expanded 2 - 4 new leaves after 4 months. Later, Calderon-Baltierra *et al*. (1993) used zygotic embryos to explore *in vitro* propagation. After overcoming problems with contamination by micro-organisms and hyperhydricity, they regenerated 180 shoots from one explant after 8 months on a medium previously developed for apricot by Mosella *et al*. (1979) supplemented with 1 mg/l BAP, 0.1 mg/l GA_3 and 0.01 mg/l NAA. Jordan *et al*. (2005) established cultures using different explants on Woody Plant Medium (WP; Lloyd and McCown 1980). Nodal explants on medium with 0.1 mg/l NAA and 1 mg/l BAP produced axillary shoots within 3 weeks, 16.7% developed callus and all explants

initiated root primordia. Under the same conditions, 40% of internodal explants produced only callus; 40% of petioles with part of the laminae still attached, developed callus and shoots. When such shoots were subcultured onto WP with 5 mg/l IBA, 3 - 5 roots developed on 46% of the shoots after 3 months.

1.7 Biogeography

G. keule is present only in Chile (Baillon 1869) from the provinces of Maule or Ñuble to Concepción or Arauco (De Candolle 1864; Espinoza 1897; Baeza 1936; Donoso 1984, 1995; Serra *et al*. 1986; Dohlen 1999), or even to Valdivia (Albert 1924). Moore (1926) listed the species amongst plants found in Paredones, the VIth administrative region, but certainly this information is not accurate, since the species is endemic to and occurs only in the VIIth and VIIIth administrative regions (Benoit 1989). However, there is agreement in the literature that *G. keule* is restricted to ravines (Serra *et al*. 1986; Donoso 1995) with difficult access (Muñoz 1986) in the coastal mountain range (Baeza 1936; Donoso 1984). More precisely, the geographical range of the species is from south of the Reloca river near Chanco, Cauquenes (35º 37' South) in the VIIth region, to Arauco in the Nahuelbuta mountain range (37º 41' South) in the VIIIth region (Muñoz 1986; San Martín and Donoso 1996; Rodríguez and Quezada 2001; Hechenleitner *et al*. 2005). The species occurs at 10 - 690 metres above sea level (masl; Rodríguez and Quezada 2001). Neger (1897) observed the species to occur mainly at 200 - 300 masl, although also at lower altitudes (50 masl). Apparently, altitude is not an important factor in species distribution (Díaz 1999). It was claimed that trees have also been found further south at Río Queule (39º20') and as far as 40°20' south (Reiche 1896; Reiche 1934, 1937), but this has not been confirmed.

The climate in the area where *G. keule* grows is typically Mediterranean, warm temperate (Le Quesne and Stark 2006), with a clear long dry season, but with humidity from the sea (Donoso 1984). Minimum temperatures are in the range 4.6 - 9.4ºC (mean 7.5), with maximum temperatures of 16.5 - 31.3ºC (mean 20^{o}C) with 14ºC (Del Fierro 1998) or 13.5ºC (Le Quesne and Stark 2006) as the annual mean. The mean air relative humidity exceeds 70% (Le Quesne and Stark 2006). Annual rainfall is 641 - 897 mm (Del Fierro 1998), possibly 950 - 1300 mm (Le Quesne and Stark 2006), mainly in the autumn and winter.

In classifying vegetation, Gajardo (1993) considered the community formed by *G. keule* and *Nothofagus obliqua* to be unique, since it includes rare species as well as species at their

natural northern boundaries. However, much of this natural habitat has been destroyed. This plant community belongs to the Concepción Deciduous Forest, in the VIIIth region, in the middle and lower slopes of the coastal mountain range, the Cordillera de la Costa. The latter is humid towards the coast and dryer towards the continent. Originally, this forest probably had a rich flora. *G. keule* also occurs in two communities in the Maulino Deciduous Forest, in the VIIth region, in the coastal mountain range on the tops, slopes and ravines near the shore. Indeed, the community of *G. keule* and *N. obliqua* is scarce. The community of *N. glauca* and *Gevuina avellana* occurs on more humid sites, on south-facing slopes. San Martín (2003) described the presence of *G. keule* in a forest stand of *N. alpina* at the latter's northernmost location on the coastal range, also in the Maulino Deciduous Forest, while Serra *et al*. (1986) summarised the species distribution as coinciding with that of the Concepción Deciduous Forest and extending further to the north in the most favourable places of the Maulino Deciduous Forest. Sixty four species, 20 of them trees, have their northern distribution limit in the coastal range of the VIIth region (San Martín 2005).

1.8 Ecology

San Martín and Donoso (1996) and Le Quesne and Stark (2006) listed those species that contribute to the vegetation of the areas where *G. keule* occurs (hygrophilic and sclerophyllic trees, shrubs, herbs, vines and epiphytes), the ecological character of which was considered as mesic by San Martín (2003). Interestingly, San Martín (2005) reported that 4 species of ferns with restricted distribution in the coastal area of the VIIth region, are especially associated with *G. keule*. Although *G. keule* grows in association with other species, it also grows in pure stands (Muñoz 1986; San Martín and Donoso 1996; Del Fierro 1998).

Neger (1897) observed trees growing in shaded sites and almost exclusively on north exposed slopes. Sepúlveda (1999) also considered the tree to be tolerant to shade with an optimum development under 55% of canopy cover. The tree is usually located in the middle layer of the canopy level, but sometimes in the upper layer, especially in pure stands (Serra *et al*. 1986). However, populations preferentially occupy south, southwest and southeast-facing slopes near streams or in ravines, although some individuals are found on north-facing slopes (San Martín and Donoso 1996). Accordingly, in Los Queules National Reserve, *G. keule* is found preferentially on south-facing slopes with humid fertile soils (Díaz 1999), from those that are thin to moderately deep (Muñoz 1986; Del Fierro 1998), and not saturated with water; trees also grow in flat sites subjected to coastal fog (Le Quesne and Stark 2006). Opazo (2006)

studied 3 localities and reported that the soils were granite based, thin, sloping, poor in phosphorus (less than 5 ppm) with a pH of 5 - 6, and more than 6% organic matter. The root depth was less than 50 cm. The same author suggested that mycorrhizae facilitate nutrient absorption by the plant.

There is agreement that the species has a fragmented distribution (San Martín 2005). Muñoz (1986) reported the trees occurred in small groups or as individuals, which was confirmed by San Martín and Sánchez (2000) who studied 14 localities each less than 3 ha in size. Stands of the tree are frequently even-aged secondary forests often with individuals represented as several stems, each commonly less than 30 cm in diameter at breast height, and grouped where the original trunk was present (Díaz 1999). Muñoz (1986) observed trees forming circles up to 2 m in diameter; Dohlen (1999) described circles 2 - 4 m in diameter with trees of 30 - 70 cm in trunk diameter. In some localities, all the individuals are shoots 10 - 20 cm in diameter arranged in a circle around each rotting stump (Figure 1.2g; Serra *et al*. 1986). This agrees with the suggestion that, at present, tree regeneration occurs mainly vegetatively (San Martín and Donoso 1996; Díaz 1999; Villegas *et al*. 2003), as in the Los Queules National Reserve (Díaz 1999). This shoot production from fired or logged trunks and from roots has enabled the species to persist in disturbed sites, although individuals lose their vigour (Le Quesne and Stark 2006).

Despite abundant fruit production, seed germination is rare (Figure 1.2c; Muñoz 1986; Serra *et al*. 1986; Villegas *et al*. 2003), or not observed in the field (San Martín and Donoso 1996; Díaz 1999; Saavedra and Matamala 2000; Le Quesne and Stark 2006). García-Gonzáles *et al*. (2008) observed 4 seedlings in one of 11 populations visited during 3 years. This fact can be attributed to anthropic disturbance, poor germination conditions because of the hardness of the endocarp (Donoso and Escobar 1985) and consumption of the fruit by rodents (Muñoz 1986; San Martín and Donoso 1996; San Martín 2005). Díaz (1999) and Maldonado (1990) suggested that *Chusquea spp*., because of its shading effect, may also contribute to poor regeneration of *G. keule* from seed. Villegas *et al*. (2003) observed some seedlings amongst stands of old trees and concluded that the genus has a strongly discontinuous regeneration pattern, *i.e.* seedlings are not produced continuously with time, and are less shade tolerant than those of two other trees, *Aextoxicon punctatum* and *Eucryphia cordifolia*, in the same stands. However, the latter authors suggested that germination is not a problem for seedling establishment as the percentage germination, although not high, is comparable to that of other tree species in the area. The present author failed to observe seedlings in this locality in 2008.

Gravity and rodents are seed dispersal vectors (Le Quesne and Stark 2006). The heavy fruit and nut indicate barochory. San Martín (2005) also noted that fruits are dispersed by gravity. However, Villegas *et al*. (2003) mentioned that the propagules, one of the largest in the Chilean flora, would hardly be dispersed by modern herbivores. The abundant edible fruit pulp may well have been consumed, and the seed dispersed, by large animals that are now extinct, as was suggested for Central American ecosystems under the anachronism idea (Janzen and Martin 1982). Central Chile was populated by megafauna in the late Pleistocene, about 11,000 years ago, with different mammals such as *Paleolama*, *Antifer* and *Equus*, with representatives of the Milodontidae and Gomphoteriidae (Labarca and López 2006).

The only mention of a direct relationship of *G. keule* with other species in its ecosystem seems to be that of Pereira *et al*. (1996), who described 25 species of lichens growing on the bark of the tree, some of them indicating the hygrophilic character of the tree. The same authors observed more lichen diversity on trunks less than 15 cm in diameter than on trunks of larger size with 14 species endemic to *G. keule* (Pereira *et al*. 2002). Martínez and Casanueva (1995) reported soil mites in stands of *G. keule*. Recently, Le Quesne and Stark (2006) observed weak trees with some cases of dieback, those most affected showing enhanced shoot production at the base of their trunks, similar to trees exposed to fire. Chlorotic foliage was caused by thrips and scale insects.

1.9 Conservation

Gomortega keule occurs within Central Chile, one of the 25 biodiversity hot spots with urgent priority for conservation on a global level (Myers *et al*. 2000). In Chile, the species was classified initially in the 'endangered' category, the closest to extinction defined after the first official national discussion on biological conservation, giving it fourth priority amongst the 11 plant species listed as most critical at that time (Benoit 1989). It was also considered endangered by the International Union for Conservation of Nature (González 1998; Walter and Gillet 1998). The species was described as a 'Natural Monument', a national legal category given to outstanding biological species for their protection, thus prohibiting the exploitation or destruction of any specimen (Ministerio de Agricultura, 1995b). Surprisingly, a later act gave permission to log trees under some circumstances (Ministerio de Agricultura, 2003). Serra *et al*. (1986) considered the species was restricted to its geographical range, because of its specific environmental requirements. However, until recent times, the tree was noticeably more abundant. These authors also suggested that as a floristic element, *G. keule*

may be a step backwards in relation to deciduous or even sclerophyll forest. Neger (1897) found *G. keule* in two localities surrounding Concepción with considerable numbers from this city to Cauquenes. Later, Baeza (1936) reported the tree was scarce at the time of his publication. Serra *et al*. (1986) recorded a major reduction in populations, some stands being in poor condition while others no longer existed. Several individuals were stump shoots, indicating the parent trees have been damaged earlier. All the trees observed by Muñoz (1986) grew from the base of trunks of logged trees. Other workers (Muñoz 1962; Rodríguez *et al*. 1983; Donoso 1984, 1995; Del Fierro 1998) reported the species as being very scarce and almost extinct. San Martín and Sánchez (2000) also concluded that populations were more abundant in the past. García-Gonzáles *et al*. (2008) suggested that human intervention has caused a reduction in the number and size of populations of the species, which, in turn, may reduce its genetic diversity. The area of occurrence of *G. keule* has been calculated to be about 116,451 ha (Del Fierro 1998). San Martín and Sánchez (2000) provided details of 22 locations, with only 2 new locations being reported by others (San Martín 2003; Stoll *et al*. 2006). In estimating the number of trees in the wild, Echeverría and Morey (2004) gave a total of 1000 individuals at 24 sites, in about 22 sub-populations each with less than 100 individuals (Hechenleitner *et al*. 2005). Only 2 of the sub-populations are in the national system of wild protected areas. Specifically, this national reserve (Reserva Nacional Los Queules) of *ca.* 150 ha was created relatively recently in 1995, in the VIIth region (Ministerio de Agricultura, 1995a). This situation is clearly not sufficient for a conservation strategy (Herrera *et al*. 2005). Besides government efforts, there are private initiatives, particularly within some forestry enterprises, to conserve populations (Morales and Calquin 2004). In this context, Sepúlveda (1999) recommended the maintenance of 55 - 65% cover of the trees with selective felling in 2 or 3 stages to protect trees younger than 7 years old growing in pine plantations.

For more than two centuries, the area where *G. keule* occurs has been affected by fires and selective logging of the better timber trees (Serra *et al*. 1986). Arroyo *et al*. (2005) also reported the coastal mountain range has been altered considerably during this time. Importantly, the natural habitat of the species in recent times has also been under permanent threat by forestry plantations of *P. radiata* which are concentrated in the same area (Rodríguez *et al*. 1983; Donoso 1984; Serra *et al*. 1986; Gajardo 1993; San Martín and Donoso 1996), as mentioned much earlier by Espinosa (1948). Likewise, Le Quesne and Stark (2006) reported that the vegetation communities where *G. keule* occurs suffer change of land use, with replacement of natural vegetation by forestry plantations of fast growing *Pinus* and *Eucalyptus*. Serra *et al*. (1986) indicated that the species seems to be especially sensitive to alteration of its environment, since these workers observed trees in very poor condition near to

exploited areas where local environmental conditions may have changed. Rodríguez *et al*. (1983) and Serra *et al*. (1986) also mentioned the extraction of *G. keule* wood as a cause of the destruction of the trees. Alteration and compaction of soil due to forestry activity, fires and illegal extraction of adult trees for charcoal and firewood, have also occurred (Le Quesne and Stark 2006). Maldonado (1990) observed clear-cutting of trees, removal of individuals and over-grazing, as factors affecting communities of *G. keule*. As mentioned, seed germination in the field is rare or absent, which can be attributed to the harvesting of fruits (Serra *et al*. 1986) and trampling by cattle (Maldonado 1990). At the Los Queules National Reserve, natural stands of the species showed signs of logging and fire damage, fruit collection by local persons, and fruit consumption and destruction by rodents (Díaz 1999; Le Quesne and Stark 2006). Díaz (1999) warned that activities, such as fruit extraction for food or scientific research, may affect regeneration and conservation of the species. Thus, the main pathway for species survival and the recovery of disturbed populations is by vegetative propagation from buds at the base of trunks, although this seems to be very slow to compensate for the destruction of populations by man (San Martín and Donoso 1996).

Some genetic studies have been performed on *G. keule* that may aid in conservation. For example, in order to investigate genetic variability, Arias (2000) extracted DNA and assessed 8 individuals using inter-simple sequence repeats (ISSR), but found little difference amongst individuals, compared to the variation in other species of Laurales such as *Peumus boldus*, *Cryptocarya alba* and *Persea lingue*. Herrera *et al*. (2005) assumed that reduction in the populations is leading to endogamy. Also using ISSR to study genetic variability within and amongst 3 populations of *G. keule*, they found a greater index of similarity compared to *Cryptocarya alba*, a Lauraceae Chilean tree that is well spread in its distribution. For *G. keule*, the authors reported *ca.* 70% of the total detectable variation was found within populations, the rest being amongst populations. They suggested a hybridization programme could be established after more detailed genetic investigation of individual trees. Using the same genetic markers (ISSR) on 223 individuals of 11 populations, García-Gonzáles *et al*. (2008) found extensive genetic variation in *G. keule*, and suggested that active gene flow occurred in the ancient continuous population. The latter authors also reported that geographic distance does not influence genetic structure. Ecological studies have been performed on population fragmentation and gene flow (Lander 2005), although the results have not been published to date. In relation to such studies, Lander *et al*. (2007) developed microsatellite markers for *G. keule*, which are expected to be valuable tools for assessing genetic diversity and breeding patterns.

The protection of all remnant populations and individuals has been strongly recommended (Hechenleitner *et al*. 2005; Herrera *et al*. 2005), as well as the restoration of damaged stands of trees and the initiation of *ex situ* conservation sites (Le Quesne and Stark 2006). The conservation of the populations at the edges of the distribution, which showed reduced genetic diversity, is a priority (García-Gonzáles *et al*. 2008). Actions involving local persons should also be considered (Herrera *et al*. 2005), as well as the owners of rural properties where the species grows (Muñoz 1986). Only one formal initiative has been accomplished in this regard (Saavedra and Matamala 2000), even though a national plan was proposed to conserve the species (Villa and Benoit 2005). In relation to *ex situ* conservation, a single plant is maintained at the Royal Botanic Garden in Edinburgh, UK, with the only other cultivated specimens being 22 trees at the Universidad Austral de Chile (Navarrete 2006).

1.10 Ethnobotany and economic botany

Although there are potential uses for *G. keule,* the tree has never been cultivated for exploitation. This may be attributed to its scarcity, isolation and difficult propagation. There is no information about the exploitation of *G. keule* by pre-Columbian populations, *i.e.* before the 16th Century, but since the earliest mention of the tree, documents have indicated some of its uses, especially its fruit. Traditional uses and knowledge of *G. keule* may disappear because of the need to conserve the tree and cultural changes in local rural communities (Muñoz and Garrido 2004a). One of the persons most informed about the uses of the plant was the Spanish author Hipólito Ruiz, who led an expedition to Peru and Chile. However, the many setbacks the expedition suffered, including a fire in Peru and a shipwreck in Portugal, both in 1785, resulted in loss of material and records from Chile, especially from Concepción (Jaramillo-Arango 1952; Ruiz 1998). In general, the use of the tree includes its exploitation for food and timber with potential for ornamental and medicinal purposes (Muñoz-Concha and Garrido 2006).

1.10.1 Fruits

The first mention of the uses of *G. keule* was in the Spanish chronicle of Jeronimo de Vivar in 1558 (Torrejón and Cisternas 2003), who described a syrup and beverage being made from the fruit. In his writings of 1674, De Rosales described the fruit as tasty and edible, either

fresh or cooked, while noting the presence of the endocarp (De Rosales, 1877). Another Jesuit, the Catalan Father Andres Febrès, a missionary in the Concepción area, wrote one of the first Spanish-Mapuche dictionaries (Febrès 1765) which included the word *queul*, stating 'a yellow fruit with a small stone within'. The abbot Molina (1782) described the tree as producing delicious fruit. Ruiz and Pavon (1798) described the fruit as non-juicy but sweet, pleasant and strong flavoured. Later, the same authors compared the shape and size of the fruit with a small hen's egg, with an attractive lustrous yellow colour (Ruiz 1998).

In work that reviewed many species as fruit plants for potential cultivation in England, Lindley (1824) described *G. keule* as the most promising of Chilean species. Reiche (1901) recommended the cultivation of the tree as the second in importance among Chilean native species after *Araucaria araucana* and indicated that if a crop was developed, it would provide fruits as valuable products (Reiche 1937). Albert (1924), a German scientist who contributed to rural and environmental affairs in the early 20th Century in Chile, listed plant foods of the country and mentioned jam of *G. keule* fruits as suitable for exporting to Europe in small quantities.

The fruits, produced in abundance in the autumn (San Martín and Donoso 1996; San Martín 2005), are well known by local people (Hechenleitner *et al*. 2005) who have used by them for years (Le Quesne and Stark 2006). Consequently, collecting fruits is considered a traditional activity (Muñoz 1986). Muñoz and Garrido (2004a) mentioned the fruits were collected by local people in April and May in some rural localities in the VIIth and VIIIth regions. Fruits were used in Concepción (Reiche 1937) and sold in abundance in the local market (Espinosa 1948), although several years later Donoso (1984) reported the fruits to be scarce but still in demand in the same markets. Serra *et al*. (1986) also mentioned that, until recently, farmers harvested and sold the fruits at markets. More recently, Maldonado (1990) purchased fruits at markets in Concepción as a source of material for germination experiments, while Saavedra and Matamala (2000) reported that local people from Tomé (VIIIth region) harvested fruits to sell.

The edible sarcocarp of the fruit has an appealing smell and yellow colour (San Martín 2005), with a soft flesh (Reiche 1937), good flavour (Moesbach 1992), juicy with a sweet pulp (Reiche 1901; Albert 1924), aromatic (Albert 1924) and savoury (Kubitzki 1993). The fruits are used to prepare jam (Espinoza 1897; Albert 1924; Reiche 1937; Muñoz 1973; Rodríguez *et al*. 1983; Donoso 1984; Saavedra and Matamala 2000; Muñoz and Garrido 2004a; Hechenleitner *et al*. 2005; Le Quesne and Stark 2006), syrup (Reiche 1901; Albert 1924; Le Quesne and Stark 2006), spirit (Saavedra and Matamala 2000; Muñoz and Garrido 2004a),

compote (Espinosa 1948) and alcoholic beverages (Hechenleitner *et al*. 2005). The fruit is consumed fresh, cooked, grilled (Albert 1924) and canned.

Using sensory evaluation, fresh fruit showed high acceptability and hardness, sweetness and aroma (Muñoz and Garrido 2004b), with low acidity, bitterness and astringency (greater in the peel than in the pulp). Soluble solids measured in the pulp of ripe fruits were, on average, 18.9 ºBx, a measurement common in the food industry to estimate sugar content. The value recorded, with other agronomic characteristics, makes the fruit an interesting potential crop for development (Muñoz *et al*. 2003). Fruits can also be consumed by cattle (San Martín and Donoso 1996). Le Quesne and Stark (2006) indicated that the seeds are edible, a fact not reported elsewhere in the literature, although Muñoz (1986) stated the seeds are soft, oily and have a taste reminiscent of the seeds of the Chilean palm, *Jubaea chilensis*.

When consumed to excess, fruits induce headaches (Ruiz 1998) or cause intoxication (Gil 1932; Santa Cruz 1932), especially when ripe and even when processed in syrup (Mariani 1965). They may be hallucinogenic (Schultes 1983) and irritant (Emboden 1979). Rodríguez (2004) mentioned that in order to prevent intoxication, local people soaked fruits in water before preparing jam. The fruits were formerly an important narcotic in Chile (Mabberley 1997), but this statement should be regarded with caution as the author was probably reviewing the literature of Schultes (1983). However, Mariani (1965) mentioned the intoxicating property of fruits is used by the *machi*, the Mapuche shaman, although he did not consider the species as being amongst the most important plants with narcotic properties for the Mapuche people. Emboden (1979) also stated that the Mapuche people utilize fresh fruit for narcotic properties, while Schultes (1983) added that they valued the tree as a psychoactive drug. Acevedo and Córdova (2004) mentioned that the fruits may act as stimulants (aphrodisiacs), although evidence and additional information were not provided. Clearly, the ethnobotanical knowledge of *G. keule* must be improved through more precise investigations.

In order to determine the possible cause of the effects of consuming fruits, Gil (1932) performed chemical analyses, and detected the presence of resins and tannins in fruits and leaves, the last compounds also being found in cells of the pericarp (Doweld 2001). The former author reported glycoalkaloids were present in fruit and suggested that they may be responsible for the intoxicating effects, but are destroyed when the fruit is cooked. In contrast, Urzúa *et al*. (1982) were unable to detect alkaloids in *G. keule*; Palma (2008) reported that alkaloids were not detected in immature or mature fruits, but in leaves. Santa Cruz (1932) mentioned processed fruit would still be intoxicating and suggested, as also reported by

Emboden (1979) and Schultes (1983), that the chemical compounds responsible for this effect may be essential oils, the latter being more potent in fresh than in dried fruits. Clearly, more research is needed to clarify the nature of the secondary products synthesised by *G. keule*.

1.10.2 Wood

Several authors described the wood as very durable and hard, with a beautiful grain (Ruiz and Pavon 1798; De Rosales 1877; Muñoz 1962; Rodríguez *et al*. 1983; Donoso 1984), especially at the base of the trunk (Albert 1924). Jaramillo-Arango (1952) and Ruiz (1998) considered the wood as fine, with a good lustre after being polished. Albert (1924) reported the wood is white-yellowish in the sapwood and dark red in the heartwood, somewhat fragrant when fresh, with regular weight, hardness and elasticity. The wood has been used for general carpentry, furniture production (Ruiz and Pavon 1798; De Rosales 1877), construction of buildings, charcoal and firewood (Espinosa 1948; Rodríguez *et al*. 1983). Reiche (1901) mentioned *G. keule* as one of the main Chilean woods with potential use for building, furniture and lathe work, especially in the central-south region of the country, while Albert (1924) also recommended it for the production of luxury furniture, veneer and inlay work. In the few places where usable trees remained, wood was used for furniture until 20 years ago (Muñoz 1986). Muñoz and Garrido (2004a) mentioned the wood was commercialised at Cauquenes, in the VIIth region of Chile. Anatomically, Stern (1955) reported the wood of *G. keule* to be diffuse-porous, with vessel diameters of 20 - 64 μm, scalariform perforation plates, with tracheids, vascular rays generally being 2 cells in width and 5 - 12 cells in height without secretory cells. This investigation was extended to include additional samples by Stern and Greene (1958).

1.10.3 Other uses

Ruiz and Pavon (1798) observed that fragrant odours are released when leaves of *G. keule* are abraded, leaves having a bitter and "balsamic" taste. Ruiz (1998) added that because of their resin content, the leaves stick to the teeth when chewed; when crushed between the fingers, they emit a fragrance suggestive of rosemary and spirits of turpentine. In judging its aromatic qualities, the same author inferred that the plant possesses healing properties, the leaves smelling like camphor to some persons (Espinosa 1948). Accordingly, Albert (1924) reported

that the leaves are aromatic and yield a medicinal oil, although Gil (1932) stated that, in the 1930s, the tree was not used in medicine. The last author also observed essence-bearing cells in the leaf parenchyma and the mesocarp, in agreement with Doweld (2001). Rodríguez (2007) extracted essential oils of *G. keule* by steam distillation and obtained an average yield of 0.73 ml/100g of dry leaves. She also reported the public's acceptance of soap and air deodorants made with these essential oils.

In the first work on the chemical constituents of the essential oil of *G. keule*, Oses *et al*. (2002) detected α-terpinene, 3-carene, camphene and 1,8 cineol, and suggested antimicrobial properties of this essential oil. In a more recent chemical characterization of *G. keule* essential oils, Bittner *et al*. (2008) detailed the constituents and proportions as 1,8 cineol (35.57 %), α-pinene (7.30 %), limonene (5.40 %), β-pinene (5.30 %), α-terpinene (7.17 %), (+)-3-carene (5.17 %), β-phellandrene (0.70 %) and other compounds (33.39 %). The same authors found that *G. keule* oil was effective against granary weevils, suggesting its potential for insect control in grain storage. Clearly, an adequate source of plant material must be developed before any commercial initiative is launched, because of the difficulties associated with conservation of *G. keule*.

The chemical composition of bark and wood has been investigated. Vilegas *et al*. (1991) detected a novel cinnamoylglucose and two coumarins, namely scoparone and scopoletin in the bark, confirming earlier results of Urzúa *et al*. (1982). However, scopoletin and other 2 coumarins were not detected in later analyses and were possible artefacts (Vilegas and Lanças 1995). Additional compounds have been detected in bark and wood, these being linear unsaturated fatty acids, steroids such as sitosterol, and unidentified phytosteroids, as well as lauric, tridecanoic, myristic and pentadecanoic acids (Vilegas and Lanças 1995).

In relation to its ornamental potential, the tree was described as one of the tallest and most splendid in Chile, with a rich green colour (Ruiz 1998) and a beautiful, leafy profile (Valenzuela 1919; Donoso and Escobar 1985; Moesbach 1992) of potential ornamental value (Muñoz 1986). Rodríguez (1988) and Riedemann and Aldunate (2003) considered the foliage and fruits, with their contrasting colours, as excellent attributes for ornamental purposes. They recommended cultivation of the tree on good quality, humid soils avoiding full sun. Reiche (1896) recommended its planting as a fruit and ornamental tree in places not subjected to freezing temperatures, while Rodríguez *et al*. (1995) suggested its use for public parks, streets and flower gardens. *G. keule* has been planted as ornamental in Concepción (Bullock and Stern 1959). The tree has potential as a fruit crop, as an ornamental, as well as for the chemical industry because of its essential oils (Del Fierro 1998).

1.11 Future perspectives

Gomortega keule is an interesting tree because of its edible fruits, excellent quality wood, essential oils and ornamental value, these attributes being emphasised from the earliest records of the 16th and 17th Centuries. Despite this, the majority of publications provide only brief descriptions or statements on divergent aspects of the tree, with a restricted number representing more formal research effort. Even more surprising is the fact that there is still no initiative aimed at the systematic cultivation of *G. keule*.

Future work should focus primarily on two main areas, notably conservation and agronomy. In conservation, basic knowledge, for example of pollination, needs to be generated to address the status of the remaining populations and individuals, and long-term *ex situ* initiatives, including *in vitro* approaches, must be implemented to complement *in situ* conservation. In agronomic terms, a considerably longer time will be required to investigate propagation, growth, phenology, fruit production and breeding, including the application of modern technologies such as molecular markers, with the aim of developing an economically important fruit crop. Specifically, *in vitro* and field propagation techniques, and the species response to agronomic management, must be addressed as a matter of priority. Exploitation of the plant for ornamental use could proceed immediately, mainly for places within its natural area of distribution, such as the city of Concepción. Since the tree may have medicinal properties, this topic is also worthy of exploration in the future. *G. keule* has been part of the life of humans of the coastal range of central Chile since ancient times. It is essential that it remains part of every-day life in the future because of its evolutionary interest and economic potential.

Chapter 2

Establishment of *in vitro* cultures of *Gomortega keule*

2.1 Introduction

A plant growth hormone is a naturally occurring compound synthesized in the plant in very low concentrations. It is transported throughout the plant to target tissues where it causes a response. Tissues or cells may have a given competence to respond. Plant hormones are usually relatively small, simple compounds which bind to specific receptors. Their synthesis or action is often affected by the presence of other hormones. The concentration of a plant hormone in a tissue or cell depends on the rate of synthesis, transport and degradation. They can be chemically modified and thus inactivated. There are five groups of plant hormones, auxins, gibberellins, cytokinins, abscisins and ethylene, although there are other substances, studied more recently, with hormone characteristics such as jasmonates, salicylates, brassinosteroids, peptides and small RNAs (Öpik and Rolfe 2005). The term "Plant Growth Regulator" (PGR) refers to artificially synthesised compounds which are active as plant hormones. Growth and development processes are usually controlled by interactions of two or more hormones (Bonga and Von Aderkas 1992), while the effect of the latter on cultured tissues depends on the species or even the genotype of the plant.

Auxins promote elongation of plant organs, mainly by cell expansion, but they also have a key influence in apical dominance, the formation of lateral roots, the development of the vascular system, parthenocarpy and senescence, their diverse actions being affected by interactions with other hormones (Öpik and Rolfe 2005). Although IAA is the principal natural auxin, it is not often used in tree tissue culture because it is unstable by autoclaving and light. Conversely, NAA and IBA are destroyed less quickly, the latter being metabolized in the tissue to IAA. Less commonly used in tissue culture of woody plants is 2,4-D as it tends to produce callusing, although is useful to induce somatic embryogenesis (Bonga and Von Aderkas 1992).

Gibberellins also promote growth, parthenocarpy, leaf senescence and delay of abscission, breaking of seed dormancy and stimulation of flowering in long day plants. Their effect in stem elongation in cereals is well known. There are over a hundred gibberellins but the biologically active ones are GA_1, GA_3, GA_4 and GA_7. Cytokinins exert similar functions as auxins and gibberellins, and probably their interaction with the latter is an important feature in determining their role. Abscisic acid generally inhibits growth and plays an important part on seed and bud dormancy and resistance to stress, as well as regulation of stomata closure. Ethylene is also considered a 'stress' hormone and, unlike other plant hormones, is a gas. It has strong effects on seedlings, produces epinasty in mature plants and has a key role in fruit ripening (Öpik and Rolfe 2005). Abscisic acid and ethylene are not used routinely in plant cultures although they can have a profound effect. However, abscisic acid has been tested to help acclimatization of *in vitro* produced plants (Pospíšilová *et al*. 2007) while substances with ethylene antagonist action have been used to overcome physiological disorders of *in vitro* cultures (Hazarika 2006).

In tissue and organ cultures, cytokinins stimulate cell division and one of their main functions is to induce adventitious shoots. Synthetic cytokinins such as Kin are widely used in plant tissue culture. Other common cytokinins applied in cultures are BAP, 2iP and Ztn, where BAP is the most active, least expensive and the only one that can be autoclaved (Bonga and Von Aderkas 1992). The concentration ratio of auxin to cytokinin in the culture medium is very important to determine the type of tissue developed. A relatively high concentration of cytokinin over auxin stimulates shoot formation, while increased auxin and low cytokinins will stimulate root formation (Öpik and Rolfe 2005). Initiation and promotion of cell division by auxins is very useful in the formation of an undifferentiated mass of cells (callus) and a balanced ratio between auxins and cytokinins favours both shoot and root formation (Sinha 2004).

The use of PGRs has facilitated the culture of plant tissues, which in turn has permitted the multiplication and production of plants. In many crop species, especially woody and vegetatively propagated plants, micropropagation is a powerful technique for the mass production of pathogen-free plants with the advantages of reduced space and less time compared to traditional methods of multiplication. For conservation purposes, *in vitro* culture allows the storage of valuable germplasm and endangered species, being considered as a complement to *in situ* conservation methods (Sarasan *et al*. 2006). The culture of cells and tissues *in vitro* also permits the genetic transformation of such material.

In vitro propagation and culture techniques are of primary importance in view of the extremely difficult conservation situation of *G. keule* and its potential as an agronomic species. Subsequently, the first step to develop *in vitro* procedures is to establish explants leading to cell and tissue proliferation and eventually organogenesis.

The objective of this chapter was to explore the establishment of *in vitro* cultures using different explant sources.

2.2 Methods

2.2.1 Plant materials

Plant material was collected from the VIIth Region of Maule and the VIIIth Region of Biobío, Chile (Table 2.1). A voucher specimen (number 153860) was deposited at the National Museum of Natural History, Santiago, Chile.

Table 2.1. Geographic coordinates and altitude of the localities where plant material were sourced.

	Locality	Latitude South	Longitude West	Altitude (metres above sea level)
North	Reserva Nacional Los Queules (National protected area)	35º 58’	72º 40’	500
	Predio Ralbún, Forestal CELCO S.A. (Private forestry farm)	36º 03’	72º 38’	540
South	Predio Pino Huacho, Forestal Tierra Chilena Ltda. (Private forestry farm)	37º 40’	73º 13’	450
	Predio Carmávida, Bosques Arauco S.A. (Private forestry farm)	37º 41’	73º 18’	300

Fruits or seeds of the current season were collected from the forest floor in May and June 2006 at Ralbún. Seeds, endocarps enclosing the true seeds, were washed in water and left to dry for 3 d. At Reserva Nacional Los Queules, fresh fruits and shoots were collected from recorded trees in March and May 2007. The material was sent to The University of

Nottingham with the required phytosanitary certificates and arrived in 17 d (received on 21st March 2007) and 7 d (received on 5th June 2007), respectively. Two vessels with axenic shoots on MS medium with 0.1 mg/l NAA and 1.0 mg/l BAP were sent from the Universidad Austral de Chile and were also received on 21st March 2007, kindly supplied by Dr Peter Seemann. A shoot from a specimen of *G. keule* cultivated at The Royal Botanic Gardens, Edinburgh (9 years old plant, accession number 1998 0718, from seed collected from the VIIth Region of Maule, Province of Cauquenes), was used as well as a source of plant material, which was received on 2nd March 2007, kindly supplied by Fiona Inches.

2.2.2 Surface sterilization of explants

Endocarps were soaked in tap water for up to 69 d and then seeds were extracted with a bench vice. All explants were sterilized with agitation (details in Table 2.2), rinsed 3 times in sterile reverse-osmosis water and placed on different media under *in vitro* conditions (details in Section 2.2.3). Embryos were extracted from cultured seeds at different times up to 86 d, or as soon as they appeared to be contaminated. Buds of the shoot received from The Royal Botanic Gardens, Edinburgh were re-sterilized from 3 d of culture onwards, as soon as any visible mycelium appeared. The 2 groups of field shoots were put in tubes with growth medium. Some shoots with fungus contamination, but clean buds, were sterilized. When buds sprouted and before mycelium infected them, the new shoots were excised, sterilized and placed on culture medium.

Table 2.2. Surface sterilization treatments applied to explants.

Explant type	Sodium hypochlorite [1] concentration (% v/v)	Time (min)
Seeds	10	30 – 60
Embryos	4	10
Shoot from Edinburgh [2]	5	10
Contaminated buds from shoot from Edinburgh [3]	5 – 10	30 – 60
First group of field shoots (received 21 March) [2, 4]	10	60
Contaminated explants from the first group [5]	10	60
Second group of field shoots (received 5 June) [2, 6]	10	60
Contaminated explants from the second group	10	60
New shoots from the second group	4	10

[1] Domestos®, Johnson Diversey Ltd., Northampton, UK.

[2] Washed in tap water and cut before sterilization.

[3] Additionally treated (once contamination appeared) with 1.5 - 2.5 ml/l propamocarb hydrochloride fungicide (Filex®, Scotts Professional, Ipswich, UK) for 30 - 60 min after each sterilization.

[4] Treated with 1.5 ml/l propamocarb for 60 min before sterilization.

[5] Additionally treated (once contamination appeared) with 2.6 ml/l propamocarb for 60 min and 4 ml/l streptomycin sulphate (Sigma-Aldrich Company Ltd., Poole, UK) for 20 min after each sterilization.

[6] Treated with 4 ml/l azoxystrobin (Amistar®, Syngenta Crop Protection UK Ltd., Whittlesford, UK) for 60 min before sterilization.

2.2.3 Explants, media and culture conditions

Explants consisted of seeds, embryos, leaves, shoots, stems (internodes and nodes with buds) and isolated buds. Three experiments to establish explants derived from groups of endocarps were performed. The first zygotic embryo culture was conducted using 20 endocarps from Ralbún with a previous soaking period of 6 d. Two consecutive cultures of embryos were established using *ca.* 210 and 160 endocarps, all from Ralbún.

Media used were according to the formulations of MS (Murashige and Skoog, 1962) and WP (Lloyd and McCown 1981), with different plant growth regulators (Table 2.3), initially based on the work of Calderón-Baltierra *et al*. (1993) and Jordan *et al*. (2005).

Table 2.3. Explants and media used for establishing cultures.

Explant	Media [1]	Plant growth regulators and other supplements (mg/l)
Seeds, embryos.	MS	NAA (0.01), BAP (1.0), GA_3 (0.0, 0.1).
	WP	NAA (0.1), BAP (1.0), GA_3 (0.0, 0.1).
	WP liquid	NAA (0.1), BAP (1.0), no agar, explant on paper bridge.
Shoots.	MS	NAA (0.01), BAP (1.0).
Leaves, stems, petioles.	MS	NAA (0.01), BAP (1.0).
	WP	NAA (0.1), BAP (1.0).
	MS (MSP_1)	NAA (2.0), BAP (0.5).
	MS (MSD_3)	IAA (2.0), BAP (1.0).
	MS (UM)	Kin (0.25), 2,4-D (2.0), thiamine HCl (9.9), nicotinic acid (4.5), pyridoxine HCl (9.5), casein hydrolysate (2000).
Buds.	MS	NAA (0.01), BAP (1.0).
	WP	NAA (0.1), BAP (1.0).
	WP liquid	NAA (0.1), BAP (1.0), no agar, explant on paper bridge.
Shoots.	WP	NAA (0.1), BAP (1.0), GA_3 (0.0, 0.1, 0.5). IBA (0.0, 1.0).

[1] All media included 10 g/l PVP-360,000, pH adjusted to 5.7 before autoclaving, 20 g/l (15 for shoots) sucrose and 8 g/l agar for MS, or 30 g/l sucrose and 6 g/l agar for WP.

The second group of endocarps (second experiment) was soaked in tap water for 4 - 6 d, and seeds were extracted and soaked in 1000 mg/l GA_3 solution for 3 d. Seeds were sterilized and placed individually in 60 ml glass tubes each with 4 ml medium. The third group of endocarps (third experiment) was soaked in tap water for 65 - 69 d, changing for fresh water every week. The seeds were extracted and soaked in 6000 mg/l GA_3 solution for 3 - 7 d, then placed in MS or WP media with neither carbohydrate nor PGRs.

An assay was established in order to initially evaluate different media (Table 2.4) using shoots longer than 0.5 cm that originated from zygotic embryos. The treatments were designed based on published work on woody species, especially from the family Lauraceae (Ahmed *et al*. 2001; Babu *et al*. 2003; Chang *et al*. 2002; Handa *et al*. 2005; Hawkins *et al*. 2007; Jordan *et al*. 2005; Kumar *et al*. 1992; Moura-Costa *et al*. 1993; Rai and Chandra 1987; Yang *et al*. 2006).

Table 2.4. Media used for evaluation of axenic shoot growth.

Basal media [1]	GA_3 (mg/l)	Auxins (mg/l)	Cytokinins (mg/l)	Others supplements
MS	-	-	-	-
MS	-	IAA 0.10	BAP 1.0	-
MS	-	IBA 0.10	BAP 1.0	-
MS	0.1	2,4-D 4.00	BAP 1.0	-
MS	0.1	NAA 0.01	BAP 1.0	Pluronic F-68 ® 0.1%
MS	0.1	NAA 0.01	BAP 1.0	Half strength MS salts
MS	0.1	NAA 0.01	BAP 1.0	-
MS	0.1	NAA 0.01	BAP 1.0	Sucrose 20 g/l
MS	0.1	NAA 0.10	BAP 1.0	-
MS	0.1	-	BAP 1.0 TDZ 0.1	-
MS	0.5	NAA 0.01	BAP 1.0	-
WP	-	-	-	-
WP	-	NAA 0.10	Kin 0.1	-
WP	-	NAA 0.10	2iP 0.1	-
WP	-	NAA 0.10	Ztn 0.1	-
WP	-	NAA 0.10	BAP 1.0	PVP 360,000 5 g/l
WP	0.1	NAA 0.10	BAP 1.0	Sucrose 30 g/l
WP	0.1	NAA 0.10	BAP 1.0	Agarose 8 g/l
WP	0.1	NAA 0.10	BAP 1.0	Phytagel 2 g/l
WP	0.1	NAA 0.10	BAP 1.0	-

[1] All media included 10 g/l PVP-360,000, pH was adjusted to 5.7 before autoclaving, 20 g/l sucrose and 8 g/l agar for MS, or 30 g/l sucrose and 6 g/l agar for WP.

Stock solutions (1 mg/ml) for NAA (Sigma-Aldrich Company Ltd., Poole, UK), IBA (Duchefa Biochemie B.V., Haarlem, The Netherlands), IAA (Sigma), 2,4-D (Sigma), BAP (Duchefa), 2iP (Sigma), TDZ (Duchefa), Kin (Duchefa), Ztn (Duchefa) and GA_3 (Sigma) were prepared by dissolving 40 mg of the PGR in a few drops of 0.1 M NaOH, then adjusting the final volume to 40 ml with reverse-osmosis water and sterilizing by passage through a 0.2 µm Sartorius Minisart® syringe filter (Sartorius Stedim UK Ltd., Epsom, UK). For IBA, GA_3 and BAP, 10 mg/l stock solutions were also prepared. All stock solutions were kept at 5°C.

The general procedure followed for the preparation of 1 litre of medium began by dissolving PVP (PVP-360,000; PVP-40,000; PVP-10,000, all from Sigma) in 500 ml warm reverse-osmosis water under constant agitation. Then, 4.4 g MS salts (Sigma) or 2.4626 g WP salts (Duchefa), sucrose (D-sucrose, Fisher Scientific UK Limited, Loughborough, UK) and activated charcoal (Sigma) were added as required. The final volume was brought to 1 litre and the pH adjusted to 5.7. Agar (8 g/l for MS medium or 6 g/l for WP medium, Sigma A7002) was added to 500 ml Duran bottles (Fisher) and the medium added. Media were autoclaved at 121ºC for 20 min. Either immediately after autoclaving, or after melting the medium in microwave oven, PGRs were added when the temperature was below 60ºC. The medium was poured into culture vessels after agitation.

All cultures were maintained at 24 - 25ºC with a 16 h photoperiod under a light intensity of 38 - 80 µmol/m^2/sec. Culture vessels used were 60 ml (2.4 cm diameter, 15 cm long) glass tubes (Sigma), plastic Universal tubes, 75 ml (4.4 cm diameter, 7.5 cm long) screw-capped round glass jars and 175 ml (5.6 cm diameter, 10.5 cm long) screw-capped round glass jars (the last two from Beatson Clark and Co. Ltd., Rotherham, UK).

Initially, 60 ml glass tubes each with 4 - 10 ml of medium were used for seeds, embryos, leaf and stem explants. Responding embryos were transferred to Universal tubes with 8 ml of medium, as well as to 75 ml jars each with *ca.* 17 ml medium. Shoots over 1 cm in height were also placed in 175 ml jars with *ca.* 30 ml of medium.

Replication details are given in Table 2.5 and Table 2.6. Observations of explants were in some cases made under a Zeiss Stemi SV60 stereomicroscope and photographs were taken using a Canon Power Shot A620 digital camera.

2.2.4 Antibiotic assay and observation of contaminants

Nine samples of bacterial-like colonies were taken from contaminated *in vitro* explants and cultured overnight in liquid LB medium (Bertani, 1951) at 28ºC in an SI50 orbital incubator (Stuart - Bibby Scientific Limited, Stone, UK). Then, 50 µl of the suspension was taken and spread on LB semi-solid medium in plastic Petri dishes (90 mm diameter and 16 mm height, Sterilin Limited, Aberbargoed, UK). Paper discs (Oxoid, Basingstoke, UK) with antibiotics were placed on the medium and cultured at 24ºC for 7 d. The amount of antibiotic in each

paper disc was 30 µg latamoxef, 15 µg rifampicin, 10 µg colistin, 60 µg erythromycin, 1.2 µg penicillin G, 1000 µg kanamycin, 5 µg vancomycin, and 100 µg for chloramphenicol, ticarcillin, clavulanate, amoxycillin, ampicillin, carbenicillin and tetracycline.

Seven cultures of the antibiotic assay, plus 4 samples from infected explants *in vitro* were Gram stained (Goldman and Green 2009). A smear was prepared, heat fixed on a glass slide and immersed in crystal violet (Pro-Lab Diagnostics, Neston, UK) for 60 sec. The slide was rinsed with water and flooded for 30 sec in Lugol's iodine solution (Pro-Lab Diagnostics), rinsed with water, immersed in methanol (Fisher) for 60 sec, rinsed with water, immersed in carbol fuchsin (Prolab Diagnostic) for 60 sec and finally rinsed with water. The samples were observed under a Nikon Optiphot microscope and photographed using a Nikon DXM1200 digital camera.

2.3 Results

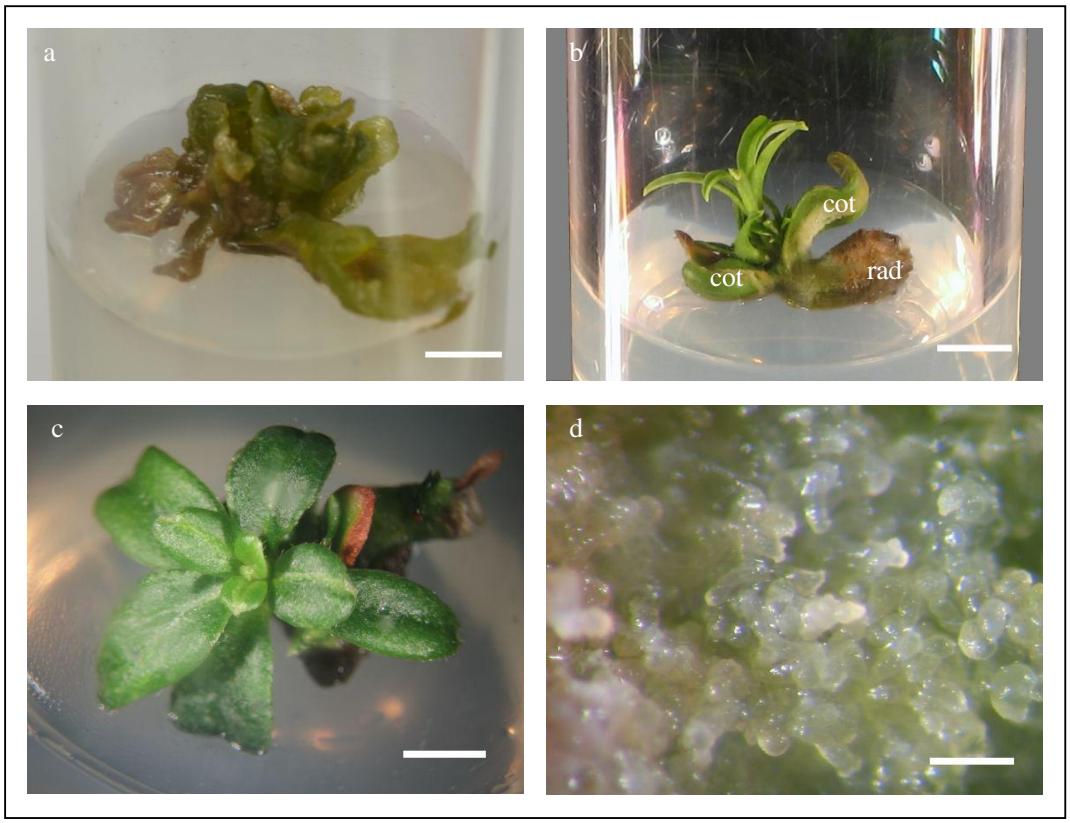
2.3.1 Establishment of zygotic embryos

The 3 experiments using explants from endocarps showed 51% of the embryos (198 of 386 seeds placed on MS or WP media) responding, with responses varying from green colour development to shoot production after 2 months of culture. Embryos that did not respond remained white, turned brown or became contaminated with fungi or bacterial-like colonies.

In the first experiment involving the culture of zygotic embryos, 8 embryos from 22 seeds showed responses (green colour, callus and shoot growth), including 2 embryos that developed abnormal shoots which continued to grow after being subdivided onto MS or WP media. However, those shoots did not show normal growth after several months (Figure 2.1a).

For cultures from the second experiment, embryos obtained from seeds which were accidentally broken (when extracted from endocarps) all became contaminated. In embryos evaluated after 58 d of culture, WP medium showed a higher proportion of embryos with shoot initiation than MS medium (Table 2.5). The time from embryo extraction to a given response was not influenced by choice of medium (Figure 2.2).

Figure 2.1. Examples of plant material in culture during the establishment period.



a) Subculture of semi-differentiated tissue (developed from a zygotic embryo) after 30 days on MS medium with 0.01 mg/l NAA and 1.0 mg/l BAP.

b) Shoot on an embryo with curved cotyledons (cot) and swollen radicle (rad) after 63 days on WP with 0.1 mg/l NAA and 1.0 mg/l BAP.

c) Shoot subcultured from material received from Universidad Austral de Chile, after 84 days on WP with 0.1 mg/l NAA and 1.0 mg/l BAP.

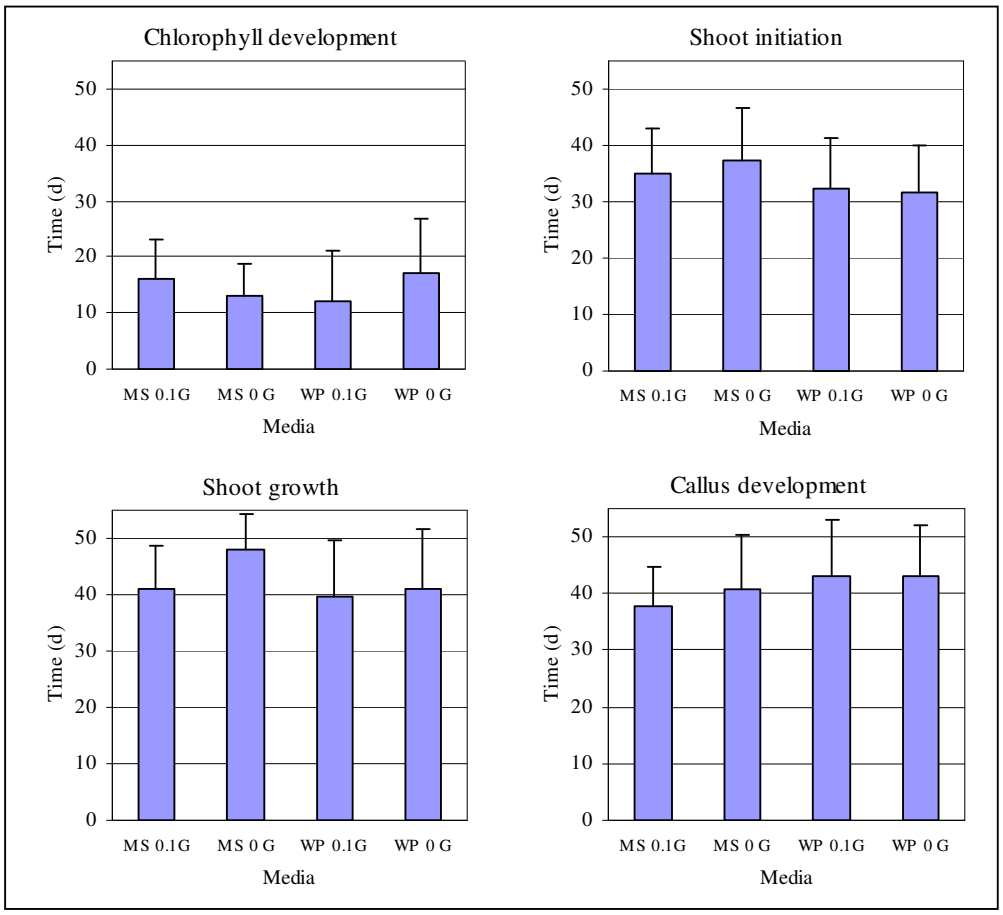
d) Friable callus on a cultured embryo after 59 days on WP with 0.1 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/l GA_3.

Scale bars = 4, 4, 3 mm and 500 µm, respectively.

For the endocarps used in the third experiment, some seeds were also accidentally broken when extracted and all their embryos were later contaminated in culture. Embryo responses for the third experiment, evaluated after 33 d of culture, are shown in Table 2.6. Again, the time from embryo extraction to a given response was not influenced by the culture medium (Figure 2.3).

Callus development seemed to be more extensive in embryos cultured on MS medium as judged by visual assessment. Some of these tissues were friable (Figure 2.1d) and were used to establish cultures in liquid media (Chapter 4).

Figure 2.2. Time living embryos required for a given response, second experiment.



Media lacked GA_3 (0 G) or were supplemented with 0.1 mg/l GA_3 (0.1 G). Only responding embryos were included to calculate the response time. Responses were evaluated during 58 days of culture.

Table 2.5. Number and percentage of embryos with different responses after 58 days of culture, second experiment.

Medium [1]	Total initial explants	Turn green		Shoot initiation		Shoot growth		Callus development		Abnormal organs [2]		Shoot > 0.5cm	
		n	%	n	%	n	%	n	%	n	%	n	%
MS 0.1 G	56	28	50.0	17	30.4	13	23.2	16	28.6	7	12.5	2	3.6
MS 0 G	59	24	40.7	19	32.2	13	22.0	20	33.9	6	10.2	2	3.4
MS Total	115	52	45.2	36	31.3*	26	22.6	36	31.3	13	11.3	4	3.5
WP 0.1 G	34	15	44.1	13	38.2	8	23.5	9	26.5	4	11.8	0	0.0
WP 0 G	52	34	65.4	26	50.0	15	28.8	17	32.7	1	1.9	6	11.5
WP Total	86	49	57	39	45.3*	23	26.7	26	30.2	5	5.8	6	7.0
TOTAL	201	101	50.2	75	37.3	49	24.4	62	30.8	18	9.0	10	5.0

[1] Media were with 0.1 mg/l GA_3 (0.1 G) or lacking GA_3 (0 G).

[2] Included hyperhydricity, fasciated and curved organs.

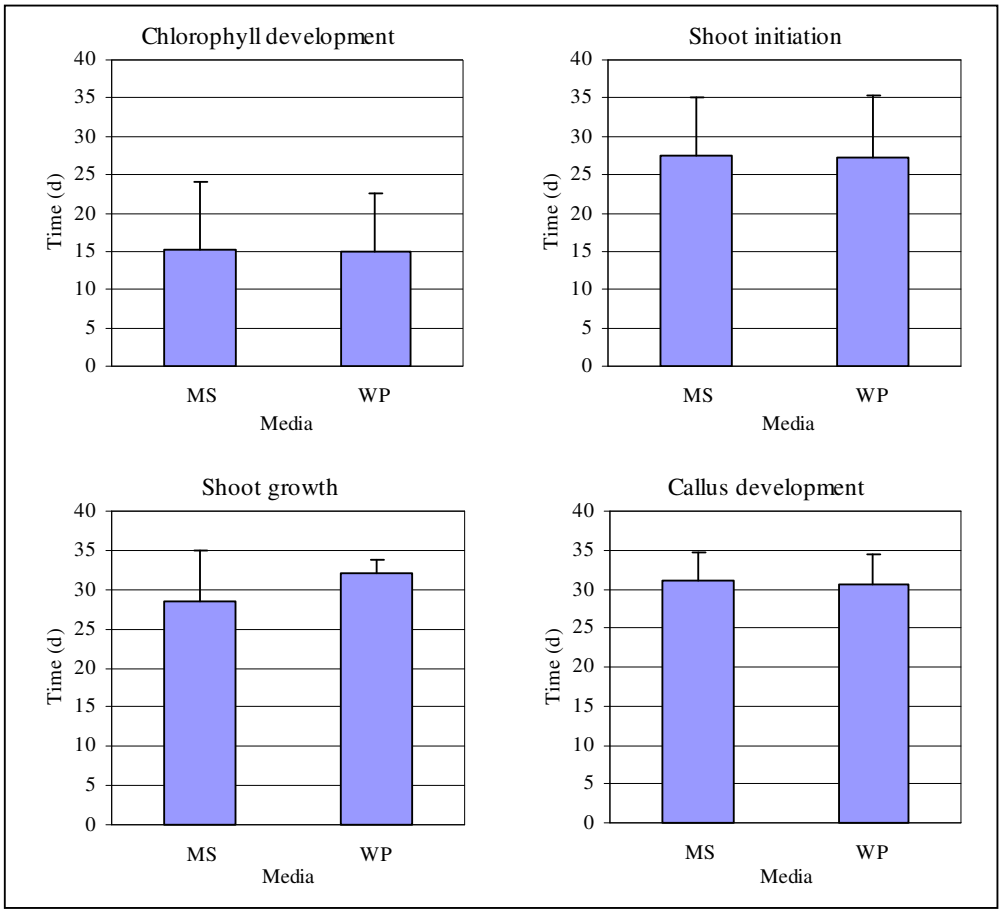
* The proportion of embryos with shoot initiation on both media was statistically different ($p<0.05$) when analysed by the Pearson Chi-square test.

Table 2.6. Number and percentage of embryos with different responses after 33 days of culture, third experiment.

Medium	Total initial explants	Total living explants		Turn green		Shoot initiation		Shoot growth		Callus development		Abnormal organs [1]	
		n	%	n	%	n	%	n	%	n	%	n	%
MS	78	39	50.0	38	48.7	11	14.1	2	2.6	6	7.7	2	2.6
WP	85	50	58.8	48	56.5	18	21.2	3	3.5	5	5.9	2	2.4
Total	163	89	54.6	86	52.8	29	17.8	5	3.1	11	6.7	4	2.5

[1] Included hyperhydricity, fasciated and curved organs.

Figure 2.3. Time living embryos required for a given response, third experiment.



The time of response was calculated using responding embryos. Responses were evaluated during 33 days of culture.

2.3.2 Establishment of non-seed explants

After 6 months, surviving explants from plant material supplied by The Royal Botanic Gardens, Edinburgh included a few stem and leaf portions with little callus production. All buds were lost due to fungal contamination. After one year, all cultures were lost due to contamination or death of non-proliferating tissues.

Cultures established using the first and second group of field shoots and their derived explants showed a very high proportion of contamination even though several treatments were carried out to sterilize them. This contamination was probably made worse by the time the material was received at the laboratory from field collection.

Buds from the first group of field shoots were excised to be placed in culture but they were almost all (>90%) lost despite repeated surface sterilization. After 5 months, surviving material comprised 2 buds from different trees, 4 stems, 1 petiole and 49 leaf explants. For the second group of field shoots, stems (*ca*. 10 cm long) without leaves were placed on MS medium with 15 g/l sucrose and lacking PGRs. When buds sprouted, the new growth was excised and surface sterilized before being placed on WP medium. This procedure gave more clean explants in culture. After 3 months, surviving material comprised 28 shoots, 35 stem and 6 petiole explants. Stem, leaf and petiole explants from field material produced limited callus, especially on WP medium.

After a year, surviving buds from the first group of field shoots did not grow and finally died. For the second group of field-collected shoots, buds from 5 different trees remained alive for more than one year. Shoots from 2 trees proliferated at very low rates while the rest were alive but showed no growth.

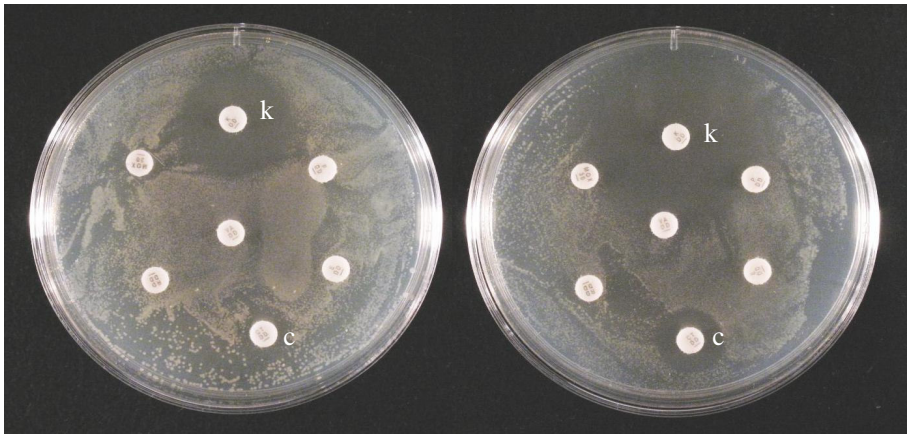
The *in vitro* cultures received from Chile produced new shoots with leaves (less than 1 cm in length) that were repeatedly subcultured (Figure 2.1c). From 4 initial clean explants, shoots proliferated slowly, but these bore curved leaves and short stems.

2.3.3 Antibiotic assay and microscopic observations

From the 9 bacterial-like colonies taken from contaminated explants and cultured in LB medium, 2 showed clear growth inhibition in the presence of 1000 µg kanamycin and, to a lesser extent, of 10 µg colistin (Figure 2.4). All contaminants stained were presumably yeasts (Figure 2.5), as a result of their shape, the presence of buds and their cell size ranging 2 - 6 µm in diameter, since bacteria are usually less than 2 µm in diameter and 8 µm in length (Prescott *et al*. 2002; Talaro and Talaro 2002; Tortora *et al*. 2001).

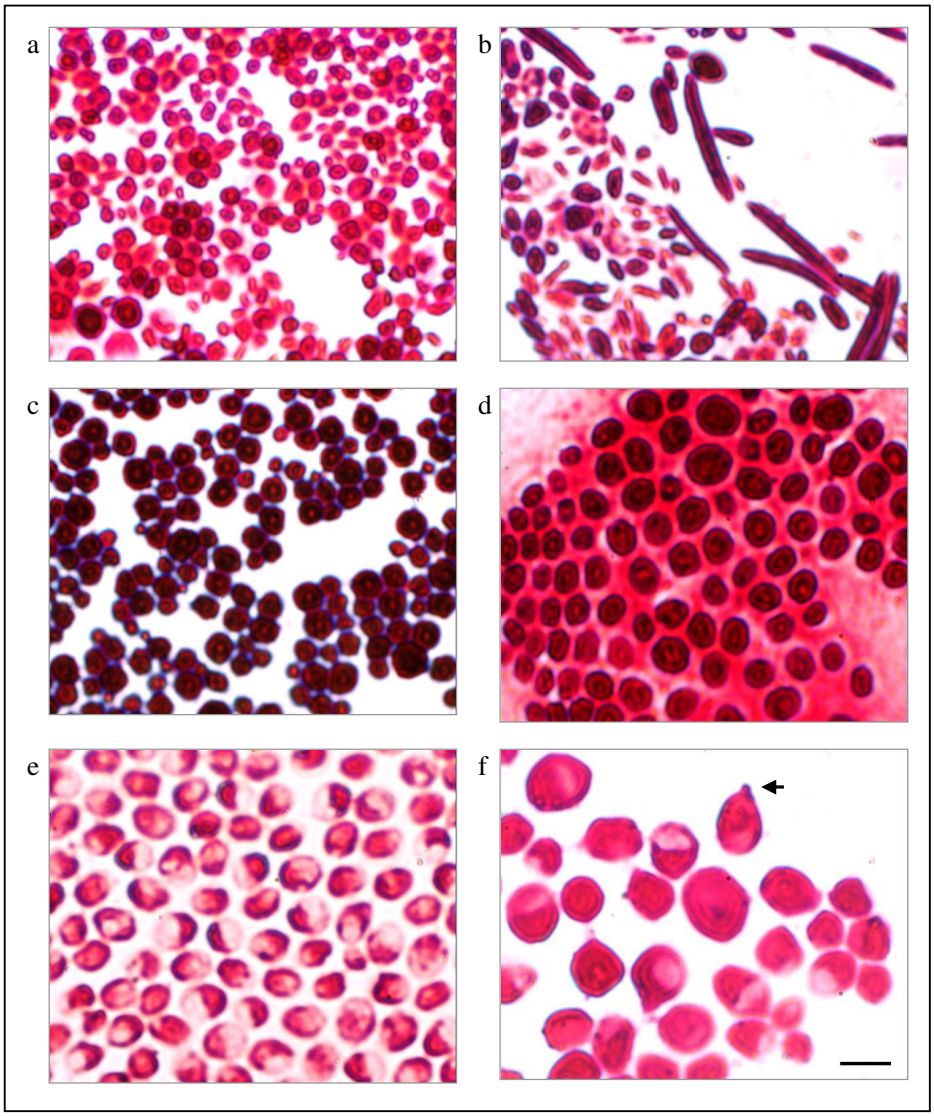
From seeds that were incubated *in vitro* without signs of contamination, embryos were excised but some became contaminated. One colony of a contaminant developed from an embryo was observed under the microscope, and was identified as a yeast species.

Figure 2.4. Yeast grown on LB medium with inhibition zones.



Inhibition zones are indicated for kanamycin (k) and colistin (c) in two cultures. The antibiotics present in the paper discs for the Petri dishes shown were 30 µg latamoxef, 15 µg rifampicin, 10 µg colistin, 60 µg erythromycin, 1.2 µg penicillin G, 1000 µg kanamycin and 5 µg vancomycin.

Figure 2.5. Yeasts from *in vitro* explants of *Gomortega keule*.



Light micrographs of yeast were taken from colonies growing from zygotic embryo (a) or non-seed material like stems or petioles (b, c, d, e, f) and showed white (a, b) or pink colour (c) in culture. They corresponded to pure cultures (c, d, e) or a mixture of species (b). Arrow shows budding. Scale bar = 5 µm (all images).

2.4 Discussion

2.4.1 Establishment of zygotic embryos

Cultured zygotic embryos showed a range of responses including the absence of any visible change, green pigmentation, swelling, callus proliferation and shoot development. This variability may reflect the genetic diversity present in a group of seeds. There was no evident trend in the response of zygotic embryos to the presence and different concentrations of PGRs. The time of response seemed to be less in embryos of the third experiment than from those from the second experiment, possibly due to the longer period that the endocarps were soaked and maybe also due to GA_3 treatment of seeds, with a greater concentration (6000 instead of 1000 mg/l) and for a longer period (3 - 7 instead of 3 d). Importantly, shoots with active growth originated from zygotic embryos enabled further experiments aimed to optimize culture conditions.

2.4.2 Establishment of non-seed explants

Sprouting of buds was observed when shoots without leaves (preferably longer than 2 cm) were incubated on culture media. Although contamination was present in a high proportion (>90%) of the cultures, the use of those new shoots seemed to be adequate to establish axenic cultures from mature trees for future research. Although Jordan *et al*. (2005) reported the successful establishment of nodal sections of *G. keule* on paper bridges with liquid WP medium, the use of paper bridges to sustain zygotic embryos, buds, stem or leaf explants was of no advantage in the present study compared to the conventional use of semi-solid medium. The time taken from collection of the field shoots to their establishment in the laboratory probably influenced their response *in vitro*. The maturity or juvenility of the donor tree may have also played a role, since it is known that establishing cultures using explants from mature trees is more difficult than using juvenile material (Andreu and Marín 2005; Barceló-Muñoz *et al*. 1999; Bonga and Von Aderkas 1992).

The lack of proliferation of organized tissues in non-seed explants is consistent with the extreme difficulty that some woody species show for stabilization under *in vitro* cultures

(McCown 2000) and the influence of the type and characteristics of the initial material on the development of cultures (Andreu and Marín 2005). *G. keule* was very difficult to stabilize, since cultures from mature plants, although successfully established *in vitro*, could not be propagated after 2 years of incubation in the present study. An exception to the non-proliferating status of the material derived from field shoots was the observation of a line of callus that originated from a bud incubated on semi-solid medium. After 8 months and with a very limited callus growth, it was transferred to liquid medium. Eighteen months later in liquid medium with a very slow growth rate, it began to show consistent callus proliferation (details in Chapter 4).

Future efforts to micropropagate *G. keule* using explants from mature trees will be crucial for the multiplication of genotypes with selected characteristics and their conservation, as has been highlighted for *Casuarina equisetifolia* (Seth *et al*. 2007), *Persea americana* (Barceló-Muñoz *et al*. 1999) and, in general, for tree species (Bonga and Von Aderkas 1992). The two hypothetical ways to achieve this in *G. keule*, given the results of the present study, will be based on the use of buds. First, the proliferation of shoots after a successful sterilization of the explant and, secondly, the proliferation of callus and the eventual occurrence of somatic embryogenesis.

2.4.3 Contamination of cultures

Microbial contamination is considered one of the major problems for plant cell culture (Kulkarni *et al*. 2007; Leifert and Cassells 2001) and is of particular importance when working with threatened taxa, often with a limited source of material from remote areas (Sarasan *et al*. 2006). Those contaminants may be introduced with the explants, during manipulations in the laboratory or by micro-arthropods, and they may proliferate immediately after the introduction to culture, or they may remain latent for a long period (Leifert and Cassells 2001; Nagy *et al*. 2005). Subsequently, small changes in the environmental conditions may allow a rapid proliferation of the contaminant (Leifert and Cassells 2001).

Consequently, in the present study with *G. keule*, contamination was a common problem when establishing cultures. Several different kinds of contaminants would have been present as judged from the variety of colours, shapes and texture of the colonies observed. Part of the contamination in cultures of *G. keule* may be attributed initially to deficient surface-sterilization of explants or inadequate manipulation, as discussed by Leifert *et al*. (1990) and

Leifert and Cassells (2001). Therefore, sterilization and manipulation of explants and cultures were checked carefully. As contamination persisted to some extent, at least part of this was presumably attributed to endophytic micro-organisms. Some embryos excised from seeds with no signs of contamination after incubation *in vitro*, became contaminated, supporting the idea that endophytic micro-organisms may have been present. Previous work on germination of *G. keule* have also attributed the microbial contamination and rotting of seeds to unidentified endogenous fungi or bacteria (Aburto 2002; Jara 2006).

The presence of yeast in cultures of *G. keule* agrees with previous reports in micropropagated plants. Yeast in the genera *Candida*, *Cryptococcus* and *Rhodotorula* have been found as contaminants in plant cultures (Leifert *et al*. 1990). Further, the presence of fungi, bacteria or yeast of endophytic origin has been reported to be common in cultured tissues (Camatti-Sartori *et al*. 2005; De Almeida *et al*. 2005; Kulkarni *et al*. 2007). In particular, Nagy *et al*. (2005) reported the endophytic presence of the yeast *Rhodotorula slooffiae* in cultures of apple, where only the combination of selected explants, surface sterilization and treatment with fungicides was successful in obtaining axenic cultures.

2.5 Conclusions

Different sources of *G. keule* material have been established *in vitro*, using zygotic embryos, bud, stem, leaf and petiole explants. The main hindrance to the establishment of cultures, particularly in explants derived from field shoots, was contamination by fungus, including yeast, probably of endophytic origin.

During the period of culture establishment, 51% of zygotic embryos showed responses such as callus and shoot production. WP medium was preferable to MS medium for the culture of zygotic embryos. Material from previous *in vitro* cultures also produced new growth. Buds from field material were also established *in vitro*, but did not show proliferation. Callus often developed from all kinds of explants, being more abundant in embryos on MS medium. Cultures often showed hyperhydricity, fasciated or curved organs.

The optimum procedure to establish *in vitro* cultures of *G. keule* with active growth was the use of zygotic embryos on semi-solid WP medium with 0.1 mg/l NAA and 1.0 mg/l BAP.

Chapter 3

Micropropagation of *Gomortega keule*

3.1 Introduction

The regeneration of a whole plant from cultured tissue is attained by shoot and root organogenesis or by somatic embryogenesis, both pathways possessing the potential for the production of large number of plants or micropropagation. Although many tree species have been propagated by these techniques, only a few cases reach a commercial scale. When callus cultures are maintained for a long time, they may tend to lose the capacity to regenerate whole plants (Kozlowski and Pallardy 1997). Moreover, undifferentiated cells tend to be genetically unstable because of mutation during the process of tissue culture, rendering genetic variation, or so called somaclonal variation, which is useful to obtain plants with new traits but undesirable for large scale production of a single genotype, where homogeneous plants are required (Kozlowski and Pallardy 1997; Öpik and Rolfe 2005). The occurence of somaclonal variation in threatened species is also of concern for conservation collections and re-establishment programs (Sarasan *et al*. 2006). Another problem of using undifferentiated callus for commercial propagation is that each species, and often each genotype, requires specific conditions (culture medium, types and concentrations of PGRs, supplements) and protocols to initiate and sustain callus in culture. The same empirical approach is valid for regeneration of plants from cultures (Kozlowski and Pallardy 1997). This may be especially critical for woody species with very specific requirements of mineral salts, PGRs and supplements (Sarasan *et al*. 2006). Even after much research, there are many woody species that have not been successfully regenerated from callus to whole plants (Kozlowski and Pallardy 1997).

Shoot tip culture is an excellent method for micropropagation. When a high cytokinin concentration is used, adventitious shoot production is promoted but shoots may be genetically more unstable because of their origin from undifferentiated tissues. Because of the

complex interaction of chemicals and plant responses, cultured tissues need to be in a competent state to respond properly. Mature tissues are often less receptive, and only when competent material has been identified can one start to optimize culture conditions (Bonga and Von Aderkas 1992). In agreement with this idea, a process of 'stabilization' is also very important in woody species during shoot proliferation with repeated subculture, where shoot growth rates increase and shoots become more uniform, with smaller leaves and narrower stems (Kozlowski and Pallardy 1997). Moreover, as indicated by McCown (2000), the inability to establish fully stabilized shoot cultures is a major cause of recalcitrance in woody and perennial crops, where growth may be erratic, with production of abnormal organs, callusing and phenolic exudation, especially in species with strong episodic flushes of growth during the growing season. Rejuvenation of cultured material occurs during stabilization and is of primary importance (McCown 2000) and, as reported by Nas *et al*. (2003), this rejuvenation may be rapidly reversed after *ex vitro* acclimatization, which is important for fruit production in trees. Shoot culture has been employed in several woody ornamental and fruit crops as well as in forest trees. A frequent physiological disorder in shoot and callus cultures is hyperhydricity, where leaves are fragile, elongated, thick and wrinkled or curled, while stems are broad and translucent. It can be reverted by changes in agar concentration and supplier, carbon source, concentration of ammonium, cold treatments (Kozlowski and Pallardy 1997) and ethylene antagonist substances (Hazarika 2006).

There are a number of additives used *in vitro* to induce or reduce specific processes or effects. Polyvinylpyrrolidone (PVP) is used to help oxidise polyphenols released into the medium by the explants (Rout *et al*. 2006) and so avoid negative effects of phenolics on the cultures. PVP has been used in *G. keule* by Jordan *et al*. (2005). According to Pan and Van Staden (1998), another important additive is ativated charcoal (AC), known to adsorb aromatic compounds such as phenolics and hormones or PGRs (auxins and cytokinins), as well as providing a dark environment which can enhance rooting. It adsorbs undesirable substances like polyphenols from the medium, avoiding inhibition of growth and morphogenesis, and can also adsorb ethylene and abscisic acid (ABA), hormones which can produce abnormal growth *in vitro*.

Root organogenesis in woody plants can be induced with an auxin, namely IBA, either in a pulse treatment or in the culture medium. Reduced strength of medium salts, dark and AC have also been explored for rooting of *in vitro* shoots in trees (Ahmed *et al*. 2001; Babu *et al*. 2003; Chang *et al*. 2002; Koubouris and Vasilakakis 2006; Rout *et al*. 2006; Sanjaya *et al*. 2006; Yang *et al*. 2006).

As reviewed by Hazarika (2006) and Pospíšilová *et al*. (2007), the acclimatization of plants produced *in vitro* implies a change in the environmental conditions. Plants *in vitro* are exposed to constant temperature, high air humidity, low irradiance and an enriched medium with sugars and PGRs, and hence they develop a heterotrophic mode of nutrition and a poor mechanism to control water loss. As a consequence, those plants do not show the morphology, anatomy and physiology that they normally develop under *ex vitro* conditions, *e.g*. failure of stomata to close, epicuticular waxes decreased, poorly developed palisade layer and weak vascular system in leaves. Thus, after transfer of the plants to *ex vitro* conditions, they need to adapt to autotrophic conditions, low air humidity, high irradiance and to correct their abnormalities (Pospíšilová *et al*. 2007). This is accomplished by gradually lowering relative humidity and giving higher levels of light, autotrophic growth and a non-aseptic environment (Hazarika 2006). Hardening may commence *in vitro* by using container lids permeable to water vapour and gases, by bottom cooling or by the use of antitranspirants in the medium (sugars in high concentration, polyethylene glycol). Further treatments may include increased irradiance and carbon dioxide concentration. Acclimatization to *ex vitro* conditions can be improved using abscisic acid and elevated carbon dioxide concentration (Pospíšilová *et al.* 2007). Rooting and acclimatization are fundamental steps in micropropagation and enable plants to be obtained suitable for establishment in the field. The success of micropropagation on a commercial scale may rely on the ability to transfer plants from *in vitro* at high survival rates. It continues to be a major bottleneck for many species (Hazarika 2006).

The establishment of axenic cultures of *G. keule* will allow, firstly, the storage and propagation of germplasm for conservation purposes and, secondly, micropropagation for plant production as a basis for further use in genetic breeding and potential commercial plant production.

The objectives of the chapter were:

1) To improve *in vitro* culture for shoot and root production.

2) To explore *ex vitro* acclimatization of *in vitro* produced plants.

3.2 Materials and Methods

3.2.1 Plant materials and culture conditions

Proliferating material obtained after the establishment phase consisted of shoots and, with slower growth, callus. The details are presented in Chapter 2.

The preparation of media and culture conditions used are also detailed in Chapter 2. Generally, 175 ml screw-capped round glass jars were used each with *ca.* 30 ml of medium. Replication details are given in Appendices 9.2 to 9.17.

3.2.2 Assessment of shoot cultures

Shoots from established cultures, on a single type of medium and of the same age were selected to set up each experiment. They were cut and prepared in a sterile plastic Petri dish in a laminar flow cabinet to give similar explants. The explants were placed in 175 ml jars, on *ca.* 30 ml of the medium chosen for the specific experiment. Also, 300 ml (7.5 cm diameter, 9.5 cm long) honey pots (Scientific Laboratory Supplies Ltd., Hessle, UK) were used. One, 3, 4 or 5 explants were inoculated in a single jar. After 50 - 100 d, the explants were evaluated by recording the weight, height, diameter, number of shoots, number of normal shoots and presence of callus. Shoots were counted if they were >5 mm in height. A shoot was considered normal when it bore at least one phenotypically normal leaf, *i.e.* over 10 mm long and 4 mm in width, generally with visible secondary veins and slightly recurved at its margins. When more than one explant per jar were evaluated, the average value of the variable measured was registered whenever possible for the analysis.

Basal media formulations (MS or WP), the use of additives (PVP and AC) in different concentrations, sucrose concentrations, agar concentrations, different PGRs in a range of concentrations, high frequency subculture (HFS), *i.e.* subculturing every week, and different temperatures were assessed. Genotypes C (culture from the Universidad Austral de Chile), S1-A, S3-60, S3-104, S3-109, S3-111, S3-214, S4-A3, S4-B6, S4-D20, S4-E1, S4-E11 and S4-F16 (originated from zygotic embryos, Chapter 2) were used in the experiments. Details of media tested are in Appendix 9.1, while details of each experiment are given in Appendices 9.2 to 9.17. Unless otherwise stated, cultures were maintained at 25ºC with a 16 h photoperiod

and a light intensity of 30 - 90 μmol/m^2/sec. For temperature and HFS experiments, WP medium was supplemented with 0.1 mg/l NAA, 1.0 mg/l BAP, 8 g/l agar, 20 g/l sucrose.

An experiment to test the effect of combinations of 5 concentrations of NAA (0, 1, 2, 3 and 4 mg/l) and BAP (0, 5, 10, 15, and 20 mg/l) in WP medium on cultured shoots of genotype S4-B6 was set up using a plastic square dish with 25 wells of 2 x 2 x 2 cm each (Sterilin Limited, Aberbargoed, UK). The 5 culture replicates were evaluated after 57 d.

For continuous and transformed data, ANOVA or t-tests were performed when homogeneous variances and normal distribution were present. When data did not satisfy those conditions, Kruskal-Wallis or Mann-Whitney tests were used, while to compare proportions, the Chi-square test was employed. Statistical tests were performed using Minitab 15.1 © 2006 or SPSS 15.0 © 2006.

3.2.3 Induction of rooting in shoots

An experiment to test rooting in shoots of genotype S4-F16 was performed by applying IBA, dark conditions, low temperature or AC according to other reports in woody species (Ahmed *et al*. 2001; Babu *et al*. 2003; Chang *et al*. 2002; Koubouris and Vasilakakis 2006; Sanjaya *et al*. 2006; Yang *et al*. 2006). Shoots were placed on WP medium (half strength salts) with 5 mg/l IBA, 10 g/l PVP-360,000, 2 g/l AC and 6 g/l agar in the light (16 h photoperiod and a light intensity of 38 - 80 μmol/m^2/sec). Shoots on full strength WP salts (same PGR and additives) were kept in the dark at 5 or 25ºC for 5 d, then transferred to jars with half strength WP salts medium, but without IBA, in the light. A fourth treatment consisted of dipping the shoot bases in a concentrated (1 g/l) IBA solution for 5 min and then placing the explants on the latter medium in the light.

A second experiment was performed using phenotypically normal shoots to test the effect of a root induction period of 6 d on WP medium with 20 mg/l IBA. Two groups of genotype S3-104 (n=15) were either maintained in the dark or light during the induction stage at 18ºC, while a third group (n=15) was treated at 5ºC in the dark. Afterwards, all shoots were cultured on WP medium with 2 g/l AC but no PGRs. For genotype S3-109, shoots were treated at 18ºC in the dark and then placed on WP medium with 2 g/l AC and full (n=13) or half (n=12) strength WP salts. A control group of shoots (n=13) was treated on WP medium lacking PGRs. The experiment was evaluated after 29 d of culture.

A further experiment was designed to test the effect of light or darkness during an induction stage of 7 d, also on medium with 20 mg/l IBA. After the treatment, shoots of genotypes S3-104 (n=20 for light, n=14 for dark) and S3-60 (n=17 for both treatments) were cultured for 31 d on WP medium with 2 g/l AC.

3.2.4 Acclimatization procedure

The lid of each 175 ml jar containing a rooted shoot cultured *in vitro* was replaced by a sterile lid with a hole plugged with sponge in order to increase gas exchange and loss of water vapour. After 2 weeks, the plant was transferred to a 9 cm diameter plastic pot with compost mix, covered with a 50 x 40 x 12 cm transparent plastic plant sleeve (Zwapak, Aalsmeer, The Netherlands), sealed and kept in a tray with 0.5 - 1 cm water in a controlled environment room at 18ºC with a 16 h photoperiod with Daylight bulbs; Osram HQI-BT 400 W/D E40 FLH1, Munich, Germany; light intensity of 110 - 130 µmol/m^2/sec and an 8 h night at 10ºC. The compost mix was made with Fine Grade Bark (William Sinclair Holdings PLC, Lincoln, UK), soil based compost (J. Arthur Bower's John Innes No. 3 Compost, Roffey Limited, Dorset, UK), perlite (Sinclair) and ground lumpwood charcoal for barbecue (9:3:2:1) following the suggestion from Clare Morter, Royal Botanic Gardens, Edinburgh. At 14 d after transfer, a first cut was made in a corner of the plastic bag, then at day 18 a second cut was made on the opposite corner. A third cut along the top of the bag was done at day 20 and a final cut around the middle of the bag at day 22. The bag was removed at day 25. The water level in the tray was checked every 2 - 4 d.

After 1 - 6 months, plants were transferred to 13 cm diameter pots or to 25 cm diameter pots depending on size and moved to the glasshouse under natural daylight, ventilating when temperature exceeded 18ºC and heating when under 10ºC. In each pot, 4 tablets of fertilizer (Osmocote Exact Tablet, Scotts Professional, Ipswich, UK) were added. Three further groups of plants were acclimatized following the same procedures, omitting the charcoal in the compost mixture.

3.3 Results

3.3.1 Micropropagation of shoots

As a general observation on the shoot proliferation experiments performed, the process of stabilization of cultures, as defined by McCown (2000), was difficult and strongly genotype dependent, taking about one year for the best cases, as in genotypes S3-104 and S3-109. Erratic responses, such as inconsistent growth and abnormalities (hyperhydricity, callusing and phenolic exudation) were commonly observed in cultured material, even from zygotic embryos (Figure 3.1).

Figure 3.1. Shoot cultures of *Gomortega keule*.

a) Development of a semi-differentiated mass of shoot-like tissues of genotype S4-E5 on MS medium with 0.01 mg/l NAA and 1 mg/l BAP.

b) Shoots growing from a zygotic embryo and callus development on the radicle of genotype S3-112 on WP medium with 0.1 mg/l NAA and 1 mg/l BAP.

c) Hyperhydric shoot with thick stem of genotype S4-A3 on MS medium with 0.1 mg/l NAA, 1 mg/l BAP and 0.5 mg/l GA_3.

Scale bar = 1 cm.

3.3.1.1 Effect of basal medium, sucrose and agar concentration

The effect of the basal medium (MS or WP) on cultures was significant for all the variables measured in genotype S4-A3, showing better responses on WP medium (Appendix 9.2). For 7 other genotypes tested, except one, the values for variables measured were higher for WP medium, although no significant differences were detected.

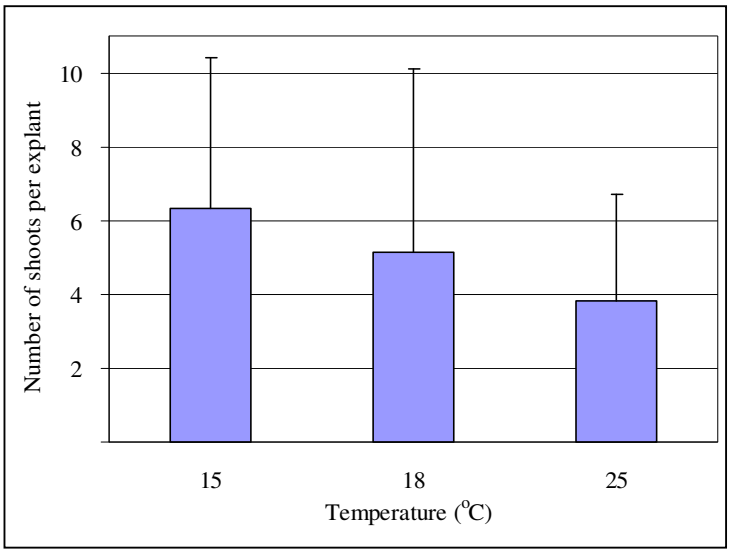
Sucrose concentration did not have a clear effect on cultures (Appendix 9.3). Only for the number of new shoots in genotype C, were differences significant, but very close to the threshold value (P=0.048). Consequently, 20 g sucrose was chosen for general culture, facilitating medium preparation with lower costs. The use of 6 or 8 g/l of agar showed no effect on cultures (Appendix 9.4). The use of 300 ml pots showed no advantage over the use of 175 ml jars.

3.3.1.2 Effect of temperature and frequency of subculture

The number of shoots produced by each explant increased as the temperature was reduced in one experiment using genotype S4-A3 (Figure 3.2), although for additional genotypes significant differences were not present (Appendix 9.5). For some genotypes that showed hyperhydric growth at 25°C (S4-A3 and C), incubation at lower temperature seemed to ameliorate the problem.

The frequency of subculture had a positive effect on the weight of explants in the 2 genotypes tested (Appendix 9.6). The values for the number of shoots and explant size were greater for HFS treatments, although significant differences were not detected.

Figure 3.2. Number of shoots produced at different temperatures.



3.3.1.3 Effect of plant growth regulators

The auxins NAA or IBA exerted no clear differences even when different concentrations were tested (Appendix 9.7). The experiment showing the only significant difference detected comprised treatments with 2.0 mg/l IBA and 0.1 mg/l NAA, and few replicates (n<6), which may not indicate a clearly different response to both PGRs. Moreover, no differences were observed on organ development or shoot quality.

Different IBA concentrations, in the range 1 - 10 mg/l, were tested on 5 genotypes but no different responses were observed, including treatments where normal shoots developed on media with AC (Appendix 9.8). A similar situation was observed in experiments where concentrations of BAP (0.1 - 5.0 mg/l) were tested on 3 genotypes (Appendix 9.9).

When including 2,4-D in the medium, callus production was enhanced compared to the presence of NAA (Appendix 9.10). The former auxin had no positive effect on shoot production, and at greater concentrations it induced necrosis. Depending on the concentration of 2,4-D, callus developed on the stem base or on the apex and leaves (Appendix 9.10).

The cytokinin BAP enhanced the number of shoots produced by genotype S4-B6 compared to 2iP (Appendix 9.11), but there was no difference on the production of shoots longer than 5 mm (Appendix 9.12). Callusing was generally stimulated by 2iP (Appendix 9.12), but explant

weight was not affected (Appendix 9.11). Callusing was increased with a greater concentration of 2iP in genotype S4-B6 (Appendix 9.13), accompanied with an increased weight of explant (Appendix 9.14), which can be attributed to the callus proliferation.

BAP also resulted in a loss of explants producing callus compared to Kin or Ztn in genotype S4-A3 (Appendix 9.12). Additionally, it produced a significantly greater number of shoots per explant in genotype S4-B6 (Appendix 9.11, Figure 3.3). The weight of explants was not affected when comparing these 3 cytokinins (Appendix 9.11).

A high concentration of Kin did not enhance callus production, although Ztn did for genotype S4-A3 (Appendix 9.13), while Ztn also showed a positive effect on the number of shoots and weight of explants for the same genotype (Appendix 9.14). The use of the cytokinin TDZ in 2 concentrations did not result in any difference on explant weight (Appendix 9.14), although some degree of bud swelling but no shoot growth were observed.

The experiment combining 2 concentrations of NAA and 3 of BAP (n=12) showed significant effects of both factors ($P<0.001$) from ANOVA for the genotype S4-A3. The combination showing the highest production of shoots after 106 d of culture was 0.1 NAA mg/l and 1.0 mg/l BAP (Figure 3.4). Necrosis of explants was not different amongst treatments (Kruskal-Wallis and Mann-Whitney tests). Previous experiments testing different concentrations of BAP in the range of 0.1 - 5.0 mg/l showed no apparent effect of concentration (Appendix 9.9), but probably the low number of replicates and the heterogeneity of the material used, hindered the detection of differences. The same occurred with the experiment using combinations of 5 concentrations of NAA and BAP, where a trend could not be observed (Figure 3.5).

The concentration of GA_3 in the range of 0 - 10 mg/l did not seem to affect cultures of 3 genotypes tested (Appendix 9.15).

Figure 3.3. Number of shoots produced with different cytokinins.

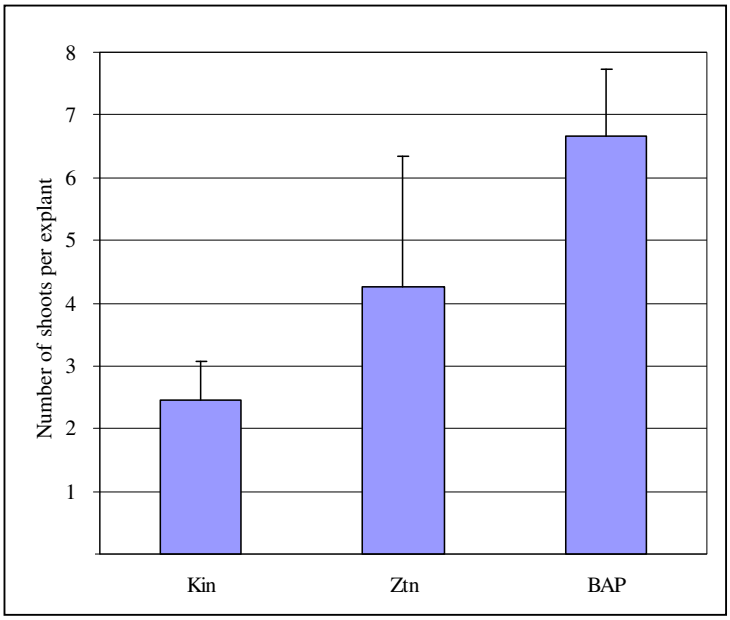


Figure 3.4. Number of shoots produced with different concentrations of NAA and BAP.

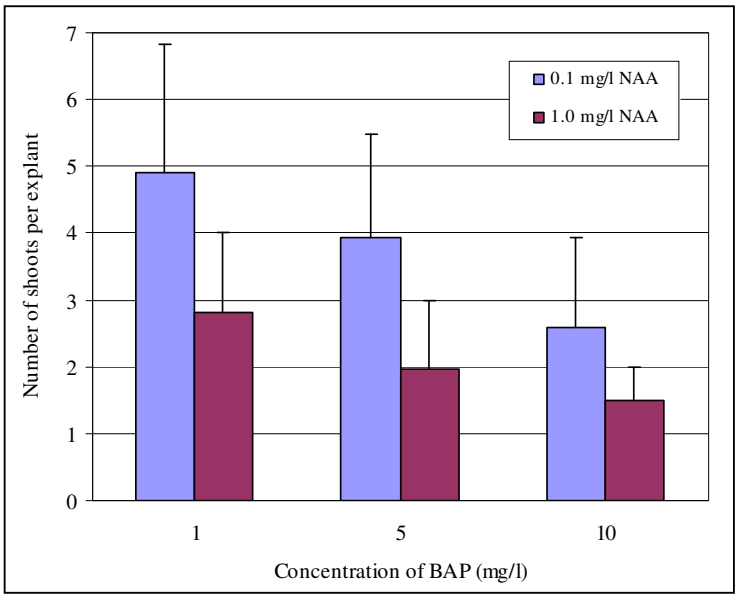
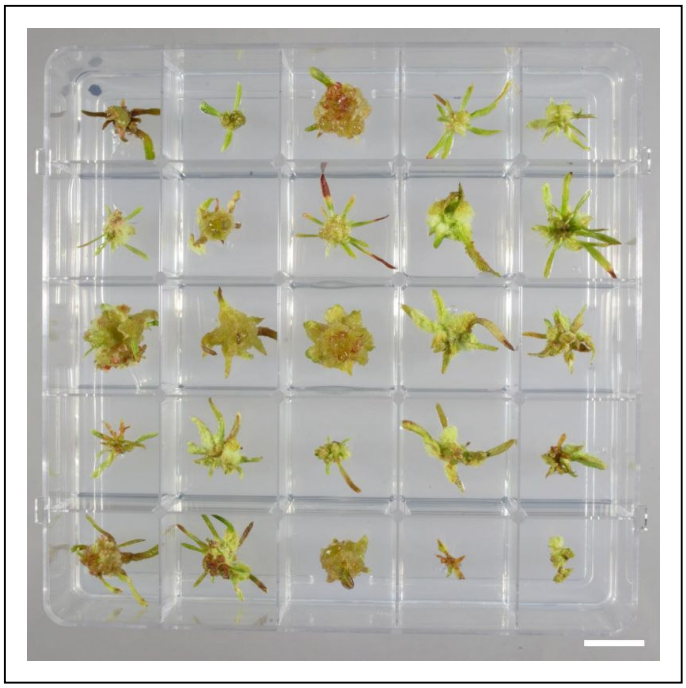


Figure 3.5. Assessment of NAA and BAP concentrations on shoots of *Gomortega keule*.



Assessment of NAA concentrations (from upper row, 0, 1, 2, 3 and 4 mg/l) and BAP concentrations (from left column, 0, 5, 10, 15 and 20 mg/l) on shoots of genotype S4-B6 cultured on WP medium. Scale bar = 1 cm.

3.3.1.4 Effect of supplements

PVP (PVP 10,000, 40,000 or 360,000) showed no clear effect on culture development (Appendix 9.16). Only the experiment for genotype S4-A3 on medium containing PVP 360,000 and AC produced significantly less weight of explants.

AC often had a negative effect on shoot proliferation, measured as the number of shoots produced by an explant, or its weight after a period of culture on semi-solid medium (Appendix 9.17). Even when the differences were not always statistically significant, most values for explants on media with activated charcoal were smaller than without AC.

For cultures of the genotype S3-60, normal shoots did not occur when media devoid of AC were used. After 96 d, 57% (n=21) of explants on media with AC developed normal shoots (Figure 3.7b) with no difference amongst 3 IBA concentrations (Kruskal-Wallis test). Similarly, 51% of explants from the same genotype in a second experiment produced normal shoots after 64 d of culture on medium with AC (n=43) with no difference between 2 IBA concentrations, compared by Chi-square test. For genotypes S1-A, S3-111, S4-E1, S4-F16, S4-E11 and S4-A3, tested with low numbers of replicates due to the scarcity of the material, normal shoots developed only on medium containing AC. However, further experiments using genotypes S3-104 and S3-109 on media devoid of PGRs, produced normal shoots regardless of the presence and concentration of AC (Figure 3.6, Appendix 9.17). In contrast, normal shoots did not grow on WP-based medium containing PGRs (0.1 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l GA_3).

Figure 3.6. Number of normal shoots produced by 2 genotypes with different concentration of activated charcoal.

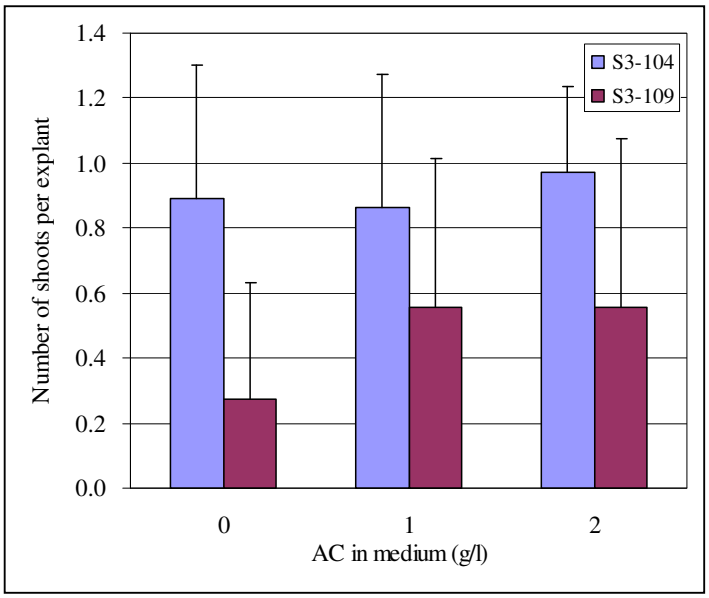
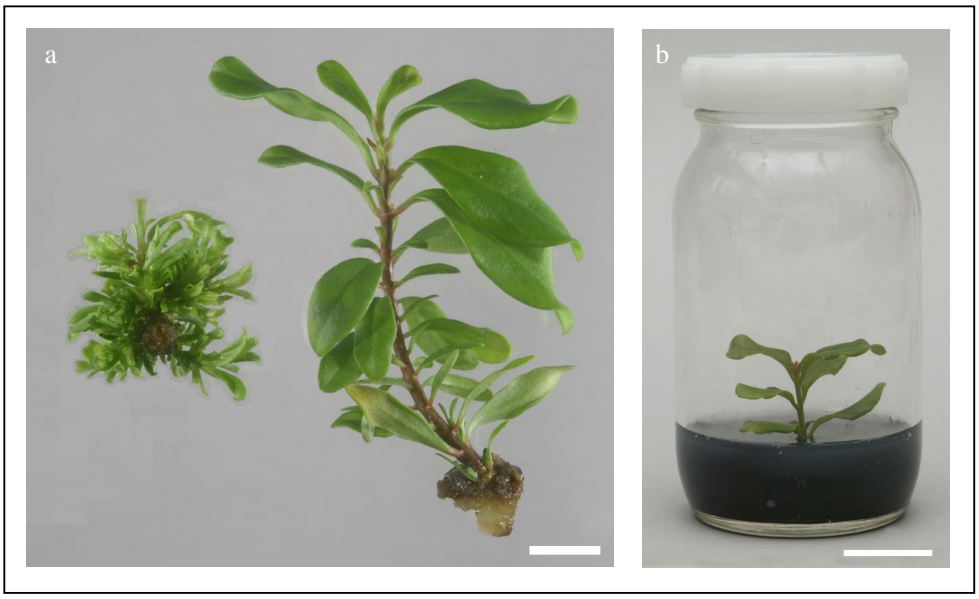


Figure 3.7. *In vitro* production of shoots of *Gomortega keule*.



a) Shoots cultured for 4 months on WP medium with 0.5 mg/l GA_3, 0.1 mg/l NAA, 1.0 mg/l BAP and 20 g/l sucrose, genotype S3-104 (left), and a shoot with normal phenotypic characteristics developed after 3 months on WP medium with 2 g/l AC, genotype S3-109 (right).

b) Shoot with normal phenotypic characteristics cultured 4 months on WP medium with 2 g/l AC, genotype S3-60.

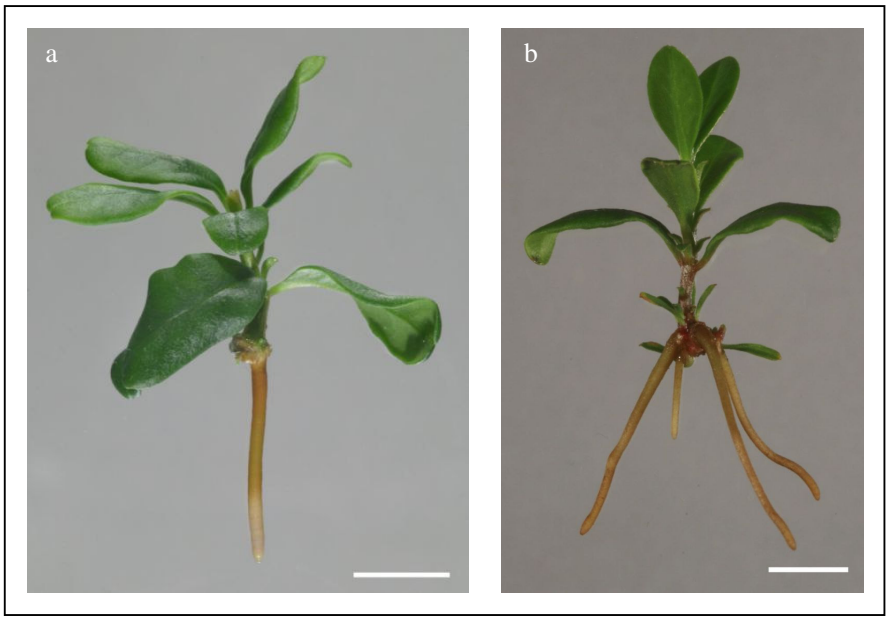
Scale bar = 1 and 2 cm.

3.3.2 Rooting of shoots

Root formation in *G. keule* was observed mainly in phenotypically normal shoots. The experiment performed with genotype S4-F16 resulted in failure to induce roots, most likely because of the poor quality of the shoots, which were abnormal. Root growth from the shoot base was not common on media devoid of AC and, in those cases, root growth seemed to be slow and the roots dark in colour. Shoots cultured on medium with AC sometimes developed a root (most commonly only one) with continuous growth and a light colour (Figure 3.8a). Such roots allowed the acclimatization of the regenerated shoots.

Some normal shoots of genotypes S3-104 and S3-109 rooted spontaneously on WP medium without PGRs, with or without AC. Up to 27% (n=26) of normal shoots in genotype S3-104 and up to 19% (n=16) in genotype S3-109 rooted spontaneously on WP medium without PGRs.

Figure 3.8. Rooted shoots of *Gomortega keule*.



a) Root developed from a shoot of genotype S3-60 cultured for 61 d on WP medium with AC.

b) Several roots developed from a shoot of genotype S3-109 after 1 week of incubation on WP medium with 20 mg/l IBA and 53 d on WP medium with AC devoid of PGRs.

Scale bars = 10 mm.

In the second experiment, genotype S3-109 showed no roots when the induction stage was not applied, while in genotype S3-104 rooting increased from 13 to 60% when the temperature in the induction stage was 18 instead of 5 °C (Chi square test, P=0.008). Differences were not detected when the induction stage was conducted in the dark (60% rooting) or light (80%), or when the shoots were cultured on full (100% rooting) or half strength WP medium (92%). Genotype S3-109 produced up to 2.7 roots per shoot, which also suggested that the number of roots produced was enhanced with IBA treatment. Chi square test was significant (P=0.0101) for rooting when genotypes S3-104 and S3-109 were compared.

The third experiment rendered no differences when the induction stage was performed in the dark or light. However, for each treatment, both genotypes assessed showed a different proportion of shoots that rooted (Table 3.1).

Table 3.1. Rooting response for 2 genotypes of *Gomortega keule* under 2 induction conditions, evaluated after 31 d of incubation.

Genotype		Induction stage [1]		P value [2]
		Light	Dark	
S3-104	Number of rooted shoots	10	10	0.2115
	Total number of shoots	20	14	
	% of rooting	50.0	71.4	
S3-60	Number of rooted shoots	2	2	-
	Total number of shoots	17	17	
	% of rooting	11.8	11.8	
	P value [2]	0.0133 *	0.0007 **	

[1] Corresponded to 7 d on medium with 20 mg/l IBA.

[2] Asterisk indicate a statistically significant difference (*, P< 0.05 and **, P<0.01) for the proportion of rooted shoots, by Chi square test.

3.3.3 Acclimatization of plants

Four groups of plants were acclimatized as *in vitro* material was available (Figure 3.9). The proportion of surviving plants ranged from 25.6% after 294 d of transfer to 65.2% after 236 d (Table 3.2). They produced new leaves and grew at irregular intervals with no synchronisation, generally showing phenotypic characteristics which were similar to those of natural plants.

Table 3.2. Survival of plants after acclimatization.

Group	% survival [1]	Time (d) [2]	n [3]
1	50.0	462	10
2	38.5	331	13
3	25.6	294	39
4	65.2	236	46

[1] Proportion of plants alive at the time of evaluation.

[2] Time at evaluation, after transfer.

[3] Initial number of plants.

Figure 3.9. Acclimatized plants of *Gomortega keule*.

a) Plants of genotype S3-104 after 5 months of acclimatization.

b) Acclimatized plant after 7 months, genotype S3-104.

Scale bars = 5 and 3 cm.

3.4 Discussion

3.4.1 Micropropagation of shoots

Given the initial scarcity of cultured material, the first experiments were performed with a limited number of replicates. This was taken into account for the interpretation of results, and may have been a source of variation. Other possible sources of variation were the different genotypes tested, the heterogeneity of the plant material (size of explant, growth rate, differentiation, hyperhydricity) and the extent of stabilization.

3.4.1.1 Effect of basal medium, sucrose and agar concentration

In genotype S4-A3, WP medium showed a better effect than MS (Appendix 9.2). Similarly, the values for the variables measured were greater for the cultures on WP medium for 7 additional genotypes, although differences were not statistically significant. The beneficial effect of WP medium is consistent with the observations during the establishment phase (Chapter 2). In *Litsea cubeba* (Lauraceae), Mao *et al*. (2000) found that for shoot propagation purposes, WP was preferable to MS as a basal medium.

Sucrose concentration did not influence cultures, and the concentration chosen (20 g/l) agreed with previous experiences in *G. keule* reported by Jordan *et al*. (2005). In rose, Rout *et al*. (2006) reported that the number of shoots was increased by reducing sucrose concentration.

Agar concentration showed no influence on shoot proliferation, but the greatest concentration tested (8 g/l) provided a more robust medium to support the explant. Hence, this concentration was generally used in cultures of *G. keule*. Additionally, a higher concentration of agar may help to prevent hyperhydricity (Hazarika 2006).

3.4.1.2 Effect of temperature and frequency of subculture

There seemed to be improved shoot production at a lower temperature (Figure 3.2, Appendix 9.5), which would agree with the temperate character of the vegetation from where *G. keule* occurs (Del Fierro 1998; Le Quesne and Stark 2006). Although in experiments using 4 different genotypes there were no significant differences relating to temperatures, the values were slightly higher at 18ºC (Appendix 9.5). Genotype C, after a year producing hyperhydric shoots at 25ºC, produced better quality shoots when cultured at 18ºC. Similar observations were for genotypes that showed improvements in the quality of shoots grown at 18ºC. This is consistent with the idea that treatments at low temperature can revert hyperhydricity (Kozlowski and Pallardy 1997).

Frequent subculture is widely used to prevent the concentration of undesirable or inhibitory substances released by plant tissues, such as phenolics (Pan and Van Staden 1998). The positive effect on explant weight observed in *G. keule* (Appendix 9.6) was possibly related to this parameter. This trend is supported by the values in the number of shoots and explant size, although differences were not significant (Appendix 9.6). Because subculture is a laborious task, a 4 or 6 week interval is probably advisable. For some trees, the repeated subculture is an effective treatment to rejuvenate or reinvigorate mature tissues. Interestingly, in some species the plants retain their maturity and flower earlier than seed-derived plants after acclimatization (Nas *et al.* 2003).

3.4.1.3 Effect of plant growth regulators

While NAA and IBA did not induce differences (Appendix 9.7), the rather low number of replicates and the heterogeneity of the material used probably did not allow detection of possible differences, as was the case for a range of IBA concentrations (1 – 10 mg/l) tested on 5 genotypes (Appendix 9.8). Therefore, and because 2,4-D was detrimental for shoot proliferation (Appendix 9.10), NAA was chosen in this study as the auxin for general culture in *G. keule*. This choice was also supported by its wide use in woody plant tissue cultures (Bonga and Von Aderkas 1992) and previous investigations with *G. keule* (Calderon-Baltierra *et al*. 1993; Jordan *et al*. 2005).

BAP was beneficial in comparison to Kin, Ztn, 2iP or TDZ (Appendices 9.11, 9.12 and 9.14) in cultures of *G. keule*. In *Prunus armeniaca*, Koubouris and Vasilakakis (2006) found

optimum production with BAP in the range of 0.5 - 2.0 mg/l, similar to 1.0 mg/l BAP being reported for shoot production in *Persea americana* (Ahmed *et al*. 2001). In the tree *Cinnamomum zeylanicum* (Lauraceae), Rai and Chandra (1987) reported that Kin induced less shoots than BAP, but such shoots were more vigorous.

In the present study, Ztn enhanced callus production in genotype S4-A3 (Appendix 9.13). Hawkins *et al*. (2007) used Ztn to propagate *Lindera melissifolia*, another endangered woody plant of the Lauraceae family, and also observed callus production. In the present study, TDZ did not promote shoot growth in *G. keule*. Thus, TDZ was considered to be inferior compared to BAP for shoot propagation. In contrast, Prakash *et al*. (2006) found that for the leguminous tree *Pterocarpus santalinus*, the combined use of BAP and TDZ induced most shoot production. Similarly, Rout *et al*. (2006) reported that TDZ was beneficial to increase axillary shoot proliferation in rose.

Regarding the different genotypes of *G. keule* tested, the induction of callus proliferation mediated by Kin or Ztn seemed to be weaker than that of 2iP. This suggested that 2iP may be useful to explore the induction of undifferentiated tissues or callus with the aim of establishing suspended cultures in liquid medium and to induce somatic embryogenesis. The auxin 2,4-D may also be included for these purposes regarding its effect on callus production. Conversely, for shoot propagation, BAP was considered the best option in terms of low callus induction and greater number of shoots. A similar situation was reported by Mao *et al*. (2000) for the tree *Litsea cubeba* (Lauraceae), where BAP was superior to Kin, 2iP or TDZ for shoot propagation.

The presence of GA_3 in the medium seemed to be not important for *G. keule*. However, given its ample use in plant cultures and the previous experience in the species (Calderon-Baltierra *et al*. 1993; Jordan *et al*. 2005), 0.5 mg/l was routinely added to the culture medium in the present experiments.

3.4.1.4 Effect of supplements

Regarding the apparent absence of beneficial effects of PVP, the costs and the time needed to prepare media, PVP-free medium was considered the best alternative. Although Jordan *et al*. (2005) included PVP 360,000 in the medium for *G. keule* cultures, they did not test its effect; Calderon-Baltierra *et al*. 1993 did not report the use of PVP. This supplement is used to

adsorb undesirable or inhibitory substances released by plant tissues *in vitro* (Pan and Van Staden 1998), as reported for shoot propagation of the tree *Cinnamomum kanehirae* (Lauraceae) by Chang *et al*. (2002).

Because AC seemed to restrict the number of shoots produced by an explant (Appendix 9.17), its presence was not desirable when high shoot proliferation was intended. However, it allowed or enhanced the production of normal shoots in the majority of genotypes tested. Accordingly, it was observed that in several genotypes, normal shoots developed only in the presence of AC, regardless of the concentration of PGRs. Also, in some genotypes (S3-104 and S3-109), although normal shoots developed in the absence of AC, they were not produced when the medium included PGRs. This suggested that the factor that triggers the production of a normal shoot is the absence of PGRs rather than the presence of AC in the medium. Additionally, normal shoots showed a marked apical dominance which was not present in shoots with rapid growth on medium with PGRs. Moreover, it can be expected that the development of normal shoots is enhanced by the sequestration of auxins by AC, although this could not be clearly detected from the experiments performed and would need further investigation. Probably, the production of normal shoots in *G. keule* is highly genotype dependent (Figure 3.6), as observed from the several genotypes tested.

AC is expected to adsorb a number of substances produced and released by explants in culture, such as phenolics and auxins (Pan and Van Staden 1998). In some tree species, strong oxidation and phenolic production have a definitive effect on cultured tissue (Kowalski and Van Staden 2001), and additives such as AC or PVP are used to overcome this problem.

3.4.2 Rooting of shoots

Rooting of normal shoots of *G. keule* was observed on medium with AC. Rout *et al*. (2006) mentioned that AC adsorbs PGRs and can improve the response for rooting in ornamental pot plants, while Babu *et al*. (2003) reported that 2 g/l AC in WP medium allowed rooting of *Cinnamomum camphora* (Lauraceae) in 30 d.

In *Casuarina equisetifolia* (Casuarinaceae), Seth *et al*. (2007) reported maximum rooting when shoots were dipped in IBA solution and then cultured in half strength salts medium without PGRs. Similarly, *Santalum album* (Santalaceae) was rooted when induced with a high concentration of IBA for 2 d followed by culture on MS-based medium with a quarter strength

salts and free of PGRs (Sanjaya *et al*. 2006). Ahmed *et al*. (2001) reported effective rooting in *Persea americana* either after a 10 min pre-treatment with a very concentrated IBA solution (1000 mg/l), or by culturing the shoots on medium supplemented with 1.0 mg/l IBA. Similarly, rooting of *G. keule* in the present study was enhanced after a treatment with IBA.

In *Pterocarpus santalinus*, Prakash *et al*. (2006) reported that the best rooting was achieved on half strength MS medium with IBA, while in *Robinia ambigua*, Guo *et al*. (2006) optimized rooting on a quarter strength MS medium with IBA and IAA. Rout *et al*. (2006) also mentioned that in *Ficus* species, rooting was reported to be induced using half strength MS salts media, and Da Silva *et al*. (2005) observed maximum rooting in *Citrus sinensis* on half strength salts medium (Murashige and Tucker 1969) with IBA. Similarly, Yang *et al*. (2006) rooted *Fortunella crassifolia* (Rutaceae) on half strength MS medium with AC. In *Prunus armeniaca*, rooting was greater than 90%, and with more roots per shoot after 10 d of induction on medium with 4 mg/l IBA and 2 weeks of culture on PGR free medium (Koubouris and Vasilakakis 2006). However, the present study on *G. keule* showed no influence of a reduced concentration of the culture medium salts on the rooting of shoots.

The experiment with genotype S4-F16 did not produce roots, which was attributed to the use of non-normal shoots, stressing the importance of the quality of the material employed. The rooting experiments clearly showed a genotype dependent response in *G. keule* (Table 3.1), as it is widely known in plant species. The percentage of rooting is very likely to increase with time for some genotypes, as small roots and root primordia were often observed at the base of shoots.

3.4.3 Acclimatization of plants

Plants were acclimatized and grown under glasshouse conditions. In all groups of acclimatized plants, some died but there was no apparent relation to a particular time or condition. As would be expected, it seemed that large shoots with actively growing roots at the time of acclimatization were important for plant survival. The proportion of surviving plants in *G. keule* was comparable to reports in other woody species, taking into account the period of evaluation. Seth *et al*. (2007) reported 58% acclimatization survival for *Casuarina equisetifolia* after 5 months, while Prakash *et al*. (2006) observed 70% survival after 5 months in *Pterocarpus santalinus*. Mao *et al*. (2000) found 30 - 90% survival in *Litsea cubeba* after 4 months, while for the shrub *Lippia filifolia*, 85% survival was recorded after 4 months

(Peixoto *et al*. 2006). In *Cinnamomum camphora*, 63% survival after 6 months was reported by Babu *et al*. (2003), 70% after 2 months in *Persea americana* (Barceló-Muñoz *et al*. 1999), 62% after 40 d in *Prunus armeniaca* (Koubouris and Vasilakakis 2006) and 85% after 2 months for *Robinia ambigua* (Guo *et al*. 2006).

The use of lids with a sponge insert for 1 - 2 weeks before acclimatization seemed not to be beneficial, but resulted in faster drying of the medium and an increased contamination. Pospíšilová *et al*. (2007) stated that hardening of *in vitro* plants may be possible by decreasing air humidity using permeable lids. However, in the present study, the lack of a positive effect and the labour involved in the procedure indicated the inconvenience of the use of such lids to harden *in vitro* produced plants. Probably, the proportion of surviving plants to acclimatization in *G. keule* may be improved with the use of good quality rooted shoots and a controlled and very gradual decrease of humidity.

The results indicate that the micropropagation of *G. keule* is a feasible method to produce plants suitable for *ex situ* conservation or reintroduction to the wild. It also represents a basic procedure for the future domestication of the species as a fruit crop tree, or for other purposes. It has been discussed (Sarasan *et al*. 2006) that the successful rooting and acclimatization of plants are fundamental for the conservation of threatened species in *ex situ* living collections and for re-establishment programmes.

3.5 Conclusions

Once axenic cultures were established, a constraint was the stabilization of the material. This required one year or more from zygotic embryos, although for several genotypes, stabilization was not accomplished. Explants derived from shoots collected in the field did not produce stabilized cultures.

Considering the responses of plant material, ease and materials consumed in the procedure, the best conditions for proliferation of shoots of *G. keule* were on WP medium with 0.1 mg/l NAA, 1.0 mg/l BAP, 8 g/l agar, 20 g/l sucrose, 18ºC and 16 h photoperiod with a light intensity of 60 μmol/m^2/sec. Subculturing the material every 4 - 6 weeks may stimulate shoot production and result in a more rapid stabilization.

The development of phenotypically normal shoots (leaves over 10 mm long and 4 mm in width) occurred on WP medium with 2 g/l AC lacking PGRs. Rooting of those *in vitro* shoots was possible for several genotypes. This was greatly stimulated with a treatment of 1 week on medium with 20 mg/l IBA, followed by culture on WP medium with 2 g/l AC but lacking PGRs. Acclimatization of the resulting plants was successful, reaching 65% survival after 8 months.

Chapter 4

Liquid cultures and somatic embryogenesis of *Gomortega keule*

4.1 Introduction

Somatic embryogenesis occurs in cultures under certain conditions and produces embryo-like structures with shoot and root poles. For woody species, research has focused on zygotic embryos, especially immature embryos, as a source of somatic embryos, although a broad range of explants have also been assessed. Embryogenic cultures are usually initiated with the use of high auxin concentrations and then can be maintained with repetitive embryogenesis at lower auxin levels. Somatic embryos may mature and subsequently develop into plants, offering a potential means for mass propagation of selected genotypes (Kozlowski and Pallardy 1997). Within micropropagation methods, somatic embryogenesis is reported to be a principal method for multiplication with the potential to produce, in a short time, the greatest number of genetically uniform plants, because the organized meristems are generally considered highly stable (Rani and Raina 2000).

Embryogenic cultures are also good material for cryopreservation in many trees as in *Quercus* (Wilhelm 2000), *Persea americana* (Efendi and Litz 2003), *Mangifera indica* (Krishna and Singh 2007) and *Citrus spp*. (Gonzalez-Arnao *et al*. 2008). In addition, embryogenic cultures have been used for genetic transformation of woody plants, as reported for *Mangifera indica* (Gómez-Lim and Litz 2004; Krishna and Singh 2007), *Coffea* (Kumar *et al*. 2006), *Citrus*, *Persea americana*, *Dimocarpus longan* and *Litchi chinensis* (Gómez-Lim and Litz 2004). Transformation of fruit trees can introduce a single trait with no further changes in a clone, which helps with the long generation time and the high levels of heterozygosity present in woody crops. Transformation in tropical trees has addressed improvement of fruit quality, control of fruit ripening, generation of parthenocarpic fruits, pest and disease resistance and alteration of tree architecture, although the regeneration of plants from selected trees in the

mature phase is a problem (Gómez-Lim and Litz 2004). Unfortunately, in olive, transformed callus has not been regenerated into plants (Pollastri 2008).

A classic treatment to induce somatic embryogenesis in many plant species is the culture of explants in medium containing the auxin 2,4-D and then to reduce or remove it from the medium to allow the expression of the process (Jiménez 2005). For several woody plants, immature zygotic embryos are excellent explants to obtain somatic embryogenesis (Giovanelli and De Carlo 2007; Ishii *et al*. 2007; Lall *et al*. 2006; Perán-Quesada *et al*. 2004; Vengadesan *et al*. 2002), although mature zygotic embryos have also been used for tree species (Iyer 2007; Moura-Costa *et al*. 1993; Thengane *et al*. 2006; Brhadda *et al*. 2008). However, the induction of somatic embryogenesis from non-seed tissues allows the propagation of selected genotypes and trees. This has been achieved in *Coffea spp*. (Samson *et al*. 2006), *Olea spp*. (Lopes *et al*. 2008) and *Sequoia sempervirens* (Korban and Sul 2007).

In coffee, indirect somatic embryogenesis was first achieved from callus after 8 or more months of culture. Later, direct somatic embryogenesis was induced within 2 - 4 months from hypocotyl explants, which then led to secondary embryogenesis and whole plant regeneration (Kumar *et al*. 2006). Also, in coffee but using young leaves, Samson *et al*. (2006) reported that the use of 2,4-D and a first stage with a medium with a high NO_3/NO_3+NH_4 ratio were key factors to induce somatic embryogenesis within 3 months. The use of 2,4-D in the dark has been reported to induce somatic embryogensis in mature zygotic embryos of *Ocotea catharinensis* (Moura-Costa *et al*. 1993), in needles of *Sequoia sempervirens* (Korban and Sul 2007), immature seeds of *Pinus armandii* (Ishii *et al*. 2007), and immature zygotic embryos of *Carica papaya* (Mishra *et al*. 2007) and *Cupressus sempervirens* (Giovanelli and De Carlo 2007). Also, 2,4-D induced somatic embryogenesis in zygotic embryos of *Eucalyptus spp*. (Le Roux and Van Staden 1991) and immature zygotic embryos of *Acacia spp*. (Beck and Dunlop 2001; Vengadesan *et al*. 2002). In *Sorbus aucuparia*, maturation of somatic embryos was achieved by Lall *et al*. (2006) in the dark and at 4°C using MS medium with maltose, but no PGRs.

Culture of endosperm has been achieved in some woody species, leading to triploid plants of potential interest for cultivation as fruit crops. Mature cultured endosperm generally leads to cell proliferation when it is initially associated with the embryo, while immature endosperm can proliferate independently; 2,4-D is usually added to the culture medium (Thomas and Chaturvedi 2008).

As somatic embryogenesis can be obtained in liquid cultures, the latter are attractive for their potential as a method of mass propagation using bioreactors. This system allows automation of the process and the mass production of somatic embryos, and then plants, in a short period of time, using reduced space. For some woody plant species, such as *Coffea arabica*, *Santalum album*, *Acanthopanax koreanum* and *Picea spp*., there is progress in the application of bioreactors, although the technique is not yet well established, especially compared to systems for non-woody plants (Yoeup and Chakrabarty 2003). The liquid medium is regarded as detrimental to many woody species and hyperhydricity is a frequent problem. Some of the challenges that the commercial use of bioreactors faces are the synchronization of the development of somatic embryos, the contamination of cultures, somaclonal variation, acclimatization of the propagules to *ex vitro* conditions and equipment costs (Yoeup and Chakrabarty 2003). For example, the commercial use of bioreactors in coffee is not yet in place because of the problems of maintaining aseptic conditions and synchrony in the induction of embryogenesis and maturation stages (Kumar *et al*. 2006).

Liquid cultures are also the preferred method to study the production of secondary metabolites in controlled conditions, being feasible for large-scale applications (Zhou and Wu 2006). Useful chemicals produced by plants, especially pharmaceuticals, are commonly found in small amounts in plant tissues, and their artificial synthesis is not economically effective. The production of these valuable secondary metabolites using *in vitro* liquid cultures would allow higher yields to be obtained of homogeneous product, avoiding the uncertainties that plants face in the field (Kim *et al*. 2002; Matkowski 2008, Zhou and Wu 2006). Callus growth in liquid media of the medicinal tree *Eucommia ulmoides* was studied by Miyanaga *et al*. (2004), who found that smaller aggregates showed greater growth rate compared to mechanically disintegrated callus.

The objectives of the chapter were:

1) To explore the use of liquid media for the culture of *G. keule*.

2) To explore somatic embryogenesis as a basis for micropropagation and clonal propagation.

4.2 Materials and Methods

4.2.1 Plant materials

Plant material was collected from the VIIth Region of Maule and the VIIIth Region of Biobío, Chile (Table 2.1). For liquid cultures, explants with callus were employed from material produced in the establishment experiments (Chapter 2). Embryogenic material had the same origin.

4.2.2 Cultures in liquid media

All media were prepared as described in Chapter 2, but autoclaved and stored in 1 litre Duran bottles without the addition of agar.

Callus induced on semi-solid MS and WP media (Section 2.2.2) from zygotic embryos or non-seed explants was placed in different liquid media (Table 4.1), with 3 replicates (flasks) per medium, on an orbital shaker at 25ºC with a 16 h photoperiod under a range of light intensities of 3 - 35 µmol/m^2/sec. Material response in liquid media was also tested using 2 concentrations of 2,4-D (0.1 and 1.0 mg/l) and AC (0 and 2 g/l), 1.0 mg/l BAP, 0.1 mg/l GA_3, 10 g/l PVP-360,000 and 20 g/l sucrose. Attempts to establish liquid cultures included non-seed explants from 10 trees and cultured material derived from 32 zygotic embryos.

Following the observation of responses of tissues in the media used to initiate cultures in liquid (Table 4.1), further media were designed with the aim of assessing callus growth (Table 4.2). These evaluations were performed by inoculating *ca.* 2 g of callus into 80 - 100 ml of liquid medium using Erlenmeyer flasks (250 ml, 6 replicates) maintained on an orbital shaker at 80 rpm, at 25 ºC with a 16 h photoperiod under a range of light intensities of 3 - 35 µmol/m^2/sec or in the dark, the media being changed every 2 weeks. Growth was evaluated by measuring the increase in fresh weight (percentage of initial weight). When cultures consisted of small embryogenic aggregates (genotypes S3-112 and B3-2853), *ca.* 50% of the medium was changed once a week. Increase in tissue volume of these small aggregates was evaluated using Erlenmeyer flasks with graduated side arms (250 ml, 8 replicates).

Table 4.1. Liquid media used to initiate callus cultures.

Basal medium [1]	Auxin (mg/l)	Cytokinin (mg/l)	Sucrose (g/l)
MS	IAA (2.00)	BAP (1.0)	30
MS	NAA (0.01)	2iP (0.1)	30
MS	NAA (0.01)	BAP (1.0)	30
MS [2]	NAA (0.01)	BAP (1.0)	30
MS	NAA (0.10)	2iP (0.1)	30
MS	NAA (2.00)	BAP (0.5)	30
UM [3]	-	2,4-D (2.0)	30
WP	IAA (2.00)	BAP (1.0)	20
WP	IBA (1.00)	BAP (1.0)	20
WP	NAA (0.01)	2iP (0.1)	20
WP	NAA (0.01)	2iP (0.1)	30
WP	NAA (0.01)	2iP (1.0)	30
WP	NAA (0.01)	BAP (1.0)	20
WP	NAA (0.10)	BAP (1.0)	20
WP	NAA (1.00)	BAP (1.0)	20
WP	NAA (2.00)	BAP (0.5)	20

[1] All media included 10 g/l PVP-360,000; pH was adjusted to 5.7 before autoclaving.

[2] Included 0.1 mg/l GA_3.

[3] MS basal medium with 0.25 mg/l kin, 9.9 mg/l thiamine HCl, 4.5 mg/l nicotinic acid, 9.5 mg/l pyridoxine HCl and 2 g/l casein hydrolysate.

Viability of cells in liquid medium was observed with the fluorescein diacetate (FDA) technique (Saunders *et al*. 1986). The FDA stock solution was prepared by dissolving 1 - 5 mg FDA (Sigma) per ml of acetone (Fisher). The FDA working solution was prepared by adding one drop of FDA stock solution to 10 ml liquid medium. A drop of FDA working solution was added to a drop of liquid culture on a glass slide, and cells were observed by UV microscopy.

Table 4.2. Liquid media used to assess callus growth.

Basal medium	Auxin (mg/l)	2iP (mg/l)	Sucrose (g/l)	PVP-360,000 (g/l)
WP	NAA (0.01)	0.1	20	10
MS	NAA (0.01)	0.1	30	10
MS	NAA (0.01)	0.1	20	10
MS	NAA (0.01)	0.1	20	0
MS	NAA (0.10)	0.1	20	0
MS	NAA (0.01)	1.0	20	0
MS	NAA (0.10)	1.0	20	0
MS	NAA (0.01)	0.5	20	0
MS	NAA (0.01)	1.0	20	0
MS	NAA (0.01)	5.0	20	0
MS	NAA (0.01)	0.0	20	0
MS	NAA (0.00)	1.0	20	0
MS	NAA (0.00)	0.0	20	0
MS	2,4-D (0.01)	0.1	20	0
MS	2,4-D (0.10)	0.1	20	0

4.2.3 Induction of somatic embryogenesis

In a first experiment to induce somatic embryos, endocarps were soaked in tap water at 4ºC for one month, then 160 seeds were extracted using a bench vice. Seeds were surface sterilized with 10% Domestos® (Johnson Diversey Ltd., Northampton, UK) for 45 min under constant agitation. Seeds were dissected and the excised embryos and endosperms were surface sterilized with 5% Domestos® for 10 min. One embryo, or 2 endosperms, was placed on semi-solid MS medium with 30 g/l sucrose, 10 g/l PVP-360,000 and 8 g/l agar in a 75 ml jar at 25ºC with a 16 h light photoperiod under a light intensity of 30 - 90 µmol/m^2/sec. Each of 7 treatments (Table 4.3) consisted of 12 - 30 embryos (total 184) and 11 - 30 endosperms (total 184). The treatments were designed considering the literature reviewed (Iyer 2007; Moura-Costa *et al*. 1993; Thengane *et al*. 2006; Brhadda *et al*. 2008; Thomas and Chaturvedi 2008) and the previous findings on callus proliferation. Cultures in the dark were wrapped in black plastic bags. Responses were evaluated at 7 months.

Table 4.3. Treatments applied to zygotic embryos to induce somatic embryogenesis.

Treatment number	Light	Induction stage			Incubation stage	
		Auxin (mg/l)	Cytokinin (mg/l)	Time (d)	Auxin (mg/l)	Cytokinin (mg/l)
1	Light	2,4-D (0.1)	2iP (0.1)	105	NAA (0.01)	2iP (0.1)
2	Light	2,4-D (1.0)	BAP (1.0)	42	NAA (0.01)	BAP (1.0)
3	Dark	2,4-D (0.1)	BAP (1.0)	8	NAA (0.01)	BAP (1.0)
4	Dark	2,4-D (1.0)	BAP (1.0)	8	NAA (0.01)	BAP (1.0)
5	Dark				NAA (0.01)	2iP (0.1)
6	Dark				2,4-D (0.1)	BAP (1.0)
7	Dark				2,4-D (0.1)	BAP (1.0) [1]

[1] Medium was supplemented with 2 g/l AC.

A second experiment to induce somatic embryogenesis was performed using shoots of genotypes S3-60 and S3-104 cultured for 7 d on MS medium containing 2 concentrations of 2,4-D (0.1 or 1.0 mg/l) and 1.0 mg/l 2iP. The explants were then transferred onto MS medium with 0.01 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/l GA_3.

A third experiment was designed after the observation of somatic embryos occurring on a shoot of genotype S4-A3 cultured on MS medium with 0.1 mg/l 2,4-D and 0.1 mg/l 2iP for 9 months. The effect of combinations of 0.1 or 1.0 mg/l 2,4-D and 0.1 or 1.0 mg/l 2iP on shoots of genotypes S3-104 and S4-A3 was evaluated after 6.5 months.

Proliferation and development of somatic embryos was attempted using 2 genotypes that showed somatic embryogenesis in previous experiments (S3-172 from establishment assessments and S3-112 from cultures in liquid medium). First, embryogenic friable callus of genotype S3-172 was placed on semi-solid MS medium at 18 and 25°C, with 0 - 10 g/l of PVP-360,000 and PGRs (0.1 mg/l GA_3, 0.01 mg/l NAA and 1.0 mg/l BAP) in the medium. After 40 d of culture and elimination of contaminated jars, cultures were evaluated (4 - 10 jars per treatment). Secondly, compact embryogenic callus of genotype S3-112 cultured in liquid MS medium with 0.01 mg/l NAA and 0.1 mg/l 2iP was placed on semi-solid MS medium with 0.01 mg/l NAA, 1.0 mg/l BAP, 0 - 0.1 mg/l GA_3 or no PGRs, using 3 explants per jar (11 or 12 jars per treatment). The response was evaluated after 3 months of culture.

Statistical procedures included ANOVA or t-test when homogeneous variances and normal distribution were present. Mann-Whitney test and Chi-square test were also employed using the softwares Minitab 15.1 © 2006 and GenStat 11 © 2008.

4.3 Results

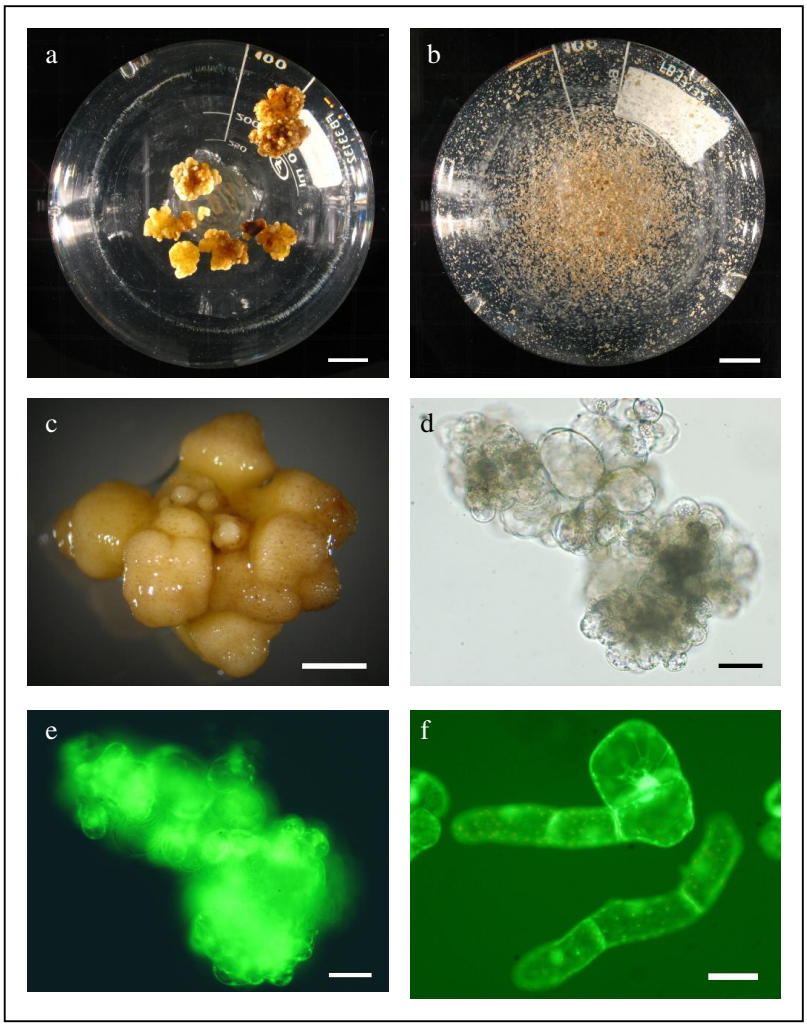
4.3.1 Cultures in liquid media

Notably, callus from genotype S3-112 developed and proliferated for more than 19 months allowing experiments to be performed in liquid media. Those calli often grew in compact aggregates (Figure 4.1a, c) and, in some cases, as smaller aggregates (Figure 4.1b) with apparently healthy cells (Figure 4.1d, e).

For several of the genotypes tested, inoculation of explants (callus from zygotic embryos) in liquid medium led to living cells which were usually observed in suspension and even dividing (Figure 4.1f). Most of the cultures in liquid media from the genotypes tested were not able to proliferate over time, and finally died. Zygotic embryos extracted from the seed and directly put in liquid media did not grow and died. The experiment using 2 concentrations of 2,4-D and AC showed no positive response.

Four attempts to establish experiments to evaluate growth using compact callus in media with or without PVP-360,000 and with 20 or 30 g sucrose were made after repetitive contamination of replicates. In the first experiment, MS medium with 20 g/l sucrose produced more growth than 30 g/l (t-test, P=0.024) and no difference was found between WP or MS basal medium and with or without PVP-360,000 after 42 d in liquid medium (Figure 4.2). Living cells smaller than plant cells observed under the microscope after staining with FDA, were found in some flasks at the end of the first experiment, indicating contamination. These cells were not further investigated, and as they did not develop mycelia, they may have been yeast. A second experiment showed no difference for the same treatments. MS medium was chosen for further culture since it stimulated the production of callus rather than well differentiated tissues previously observed on MS semi-solid media (Chapter 2). The use of 20 g/l of sucrose without PVP was employed because of the findings in the first experiment, easier media preparation and reduced costs.

Figure 4.1. Callus cultures in liquid media.



a) Callus aggregates of genotype S3-112 in a 250 ml Erlenmeyer flask.

b) Small callus aggregates of genotype S3-112.

c) Compact callus of genotype S3-112.

d) Small aggregate of living cells observed under microscope with bright field illumination, genotype S3-112.

e) Same as d), observed under microscope with UV lamp using FDA staining.

f) Living cells of genotype S4-B6.

Scale bars = 1 cm (a and b), 1 mm (c), 20 µm (d and e) and 50 µm (f).

When evaluating 2 concentrations of NAA and 2iP, differences were found between NAA concentrations (two-way ANOVA, P=0.008 for NAA), but not for 2iP after 14 d of culture (Figure 4.3). As one of the treatments was contaminated and lost, concentrations of NAA and 2iP were compared in pairs after 42 d of culture in an additional experiment. There was no difference for NAA, while a weak difference was seen with 2iP (t-test, P=0.050). Liquid MS medium with 0.01 mg/l NAA and 0.1 mg/l 2iP was regarded as optimum.

Figure 4.2. Fresh weight increment (%) with different basal media, and sucrose and PVP concentrations.

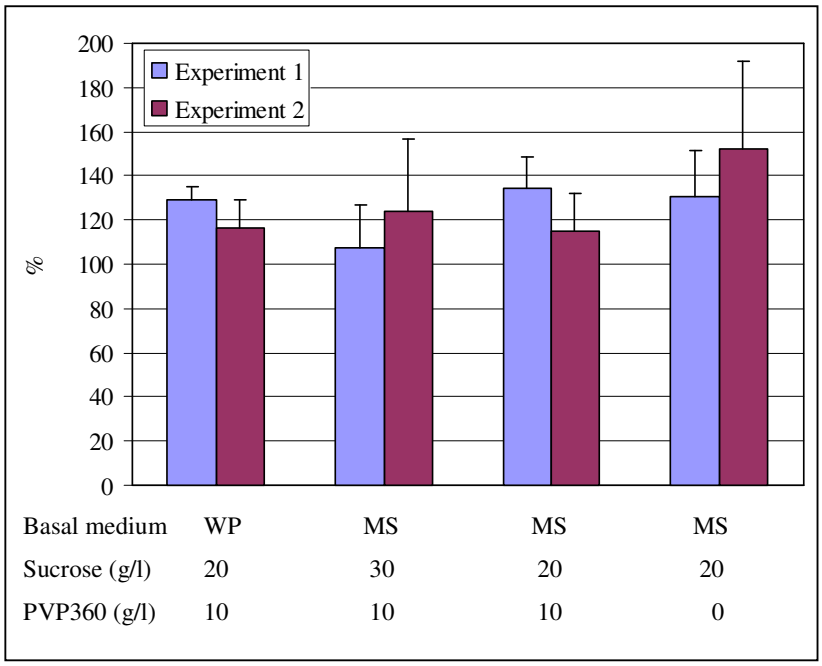
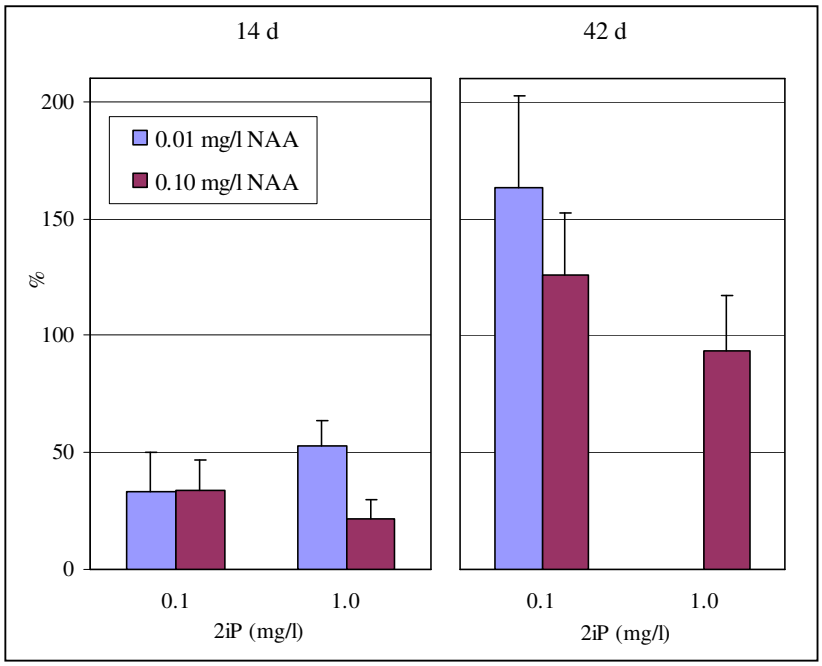


Figure 4.3. Fresh weight increment (%) with 2 concentrations of NAA and 2iP.



The treatment with 0.01 mg/l NAA and 1.0 mg/l 2iP at 42 d was lost due to contamination.

Concentrations of 2iP from 0.1 to 5.0 mg/l influenced the growth of liquid cultures (ANOVA, $P<0.001$) with the greatest growth at the minimum concentration (Figure 4.4). Combinations of NAA (0 and 0.01 mg/l) and 2iP (0 and 0.1 mg/l) (n=5 or 6) showed a significant effect for 2iP ($P<0.001$) after 44 d of culture, from two-way ANOVA (Figure 4.5).

Figure 4.4. Fresh weight increment (%) with different concentrations of 2iP.

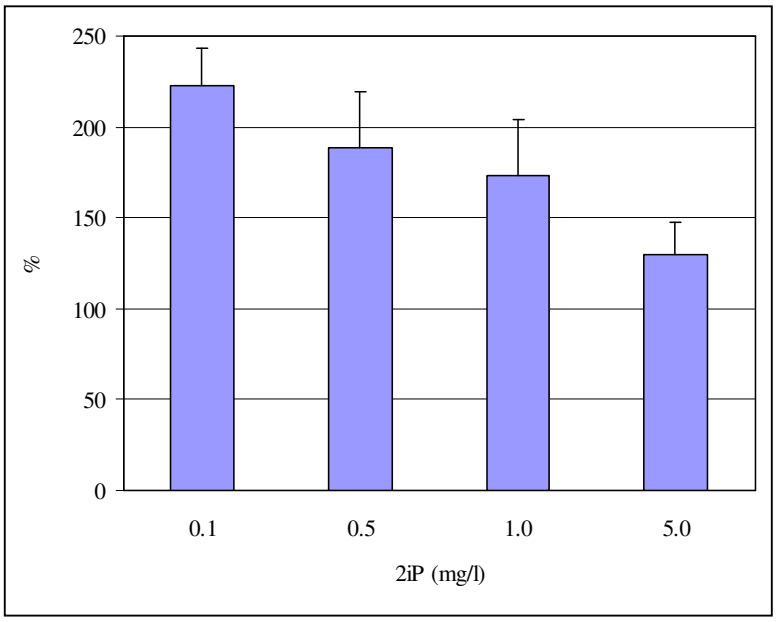
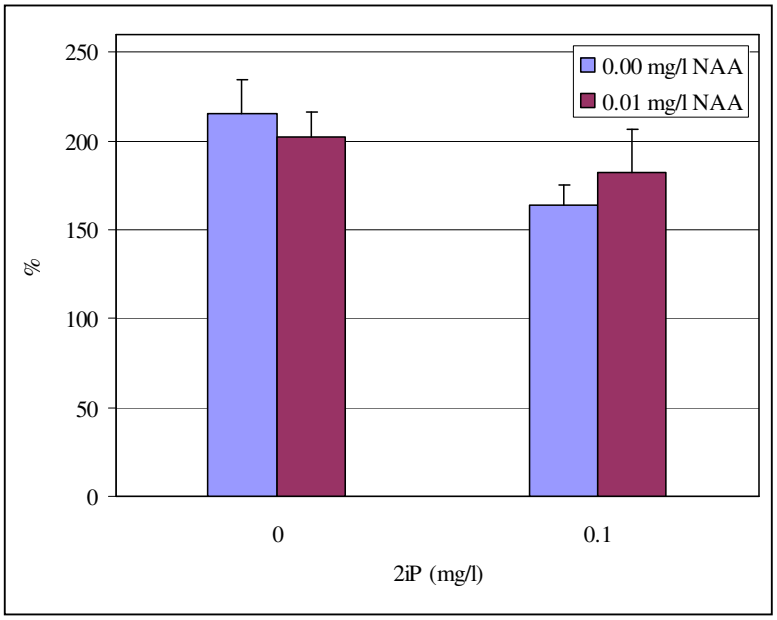


Figure 4.5. Fresh weight increment (%) with and without PGRs.



The concentrations of 2,4-D and 2iP showed significant differences (ANOVA, $P<0.001$), where the treatment using 0.1 mg/l 2,4-D and 0.1 mg/l 2iP showed reduced growth (Figure 4.6). No differences were present among the rest of the treatments under Tukey's confidence intervals (95%).

Compact callus was also obtained when somatic embryos of genotype S3-172 cultured on semi-solid medium were transferred to liquid medium. The calli showed constant proliferation over more than 12 months, and a very similar morphology to those of genotype S3-112, although the production of small aggregates was not observed.

Small aggregates originated from compact callus of genotype S3-112, and proliferated actively during more than 18 months in MS liquid medium with 0.01 mg/l NAA and 0.1 mg/l 2iP, increasing the tissue volume in the culture at a rate of 0.1 ml/d (Figure 4.7). Small aggregates were also obtained from callus that originated from a bud of a field-collected shoot from genotype B3-2853. After 18 months in MS liquid medium with 0.01 mg/l NAA and 0.1 mg/l 2iP, proliferation became stable, the cells frequently showing irregular and elongated shapes, as well as being highly vacuolated and large in size (Figure 4.8).

Figure 4.6. Fresh weight increment (%) with different concentrations of auxins.

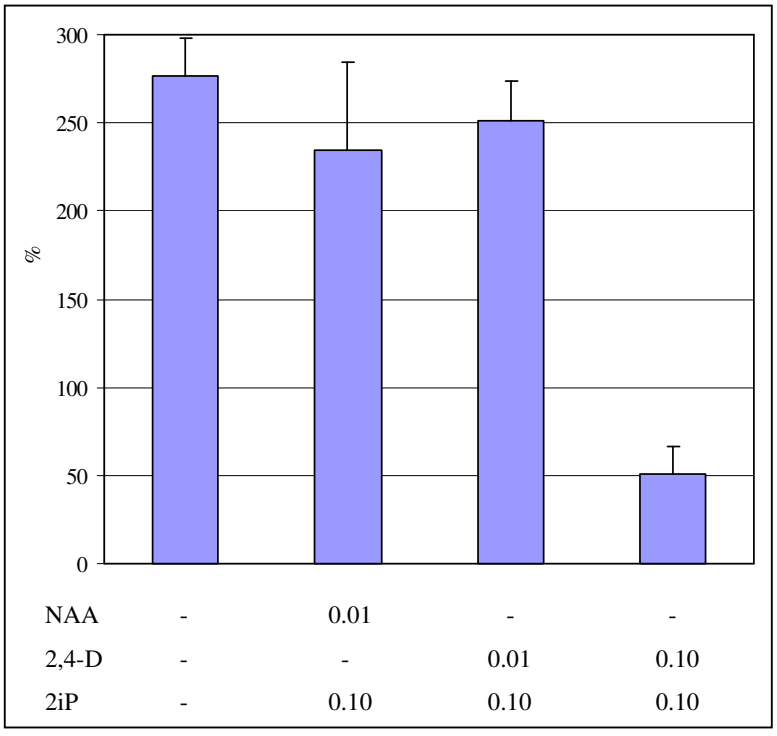


Figure 4.7. Growth of small aggregates of genotype S3-112.

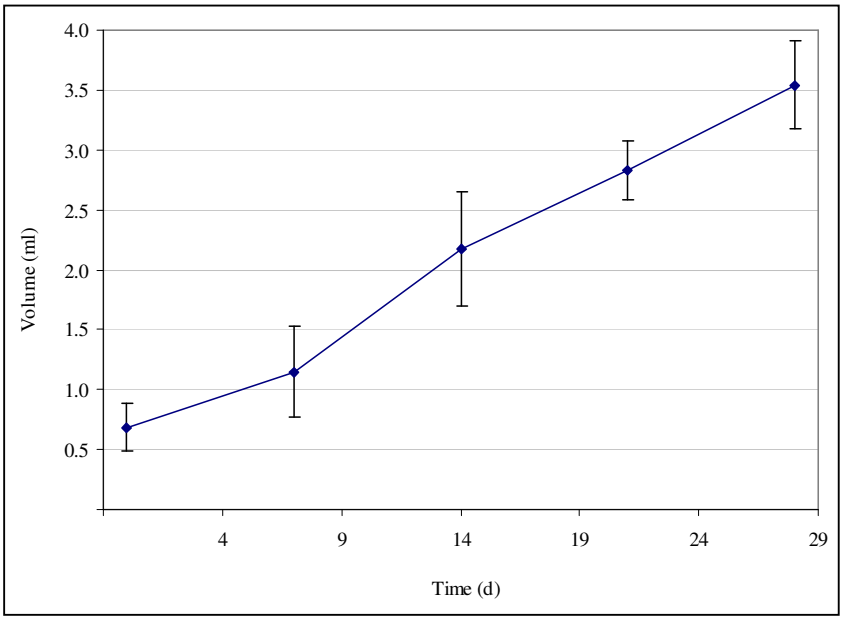
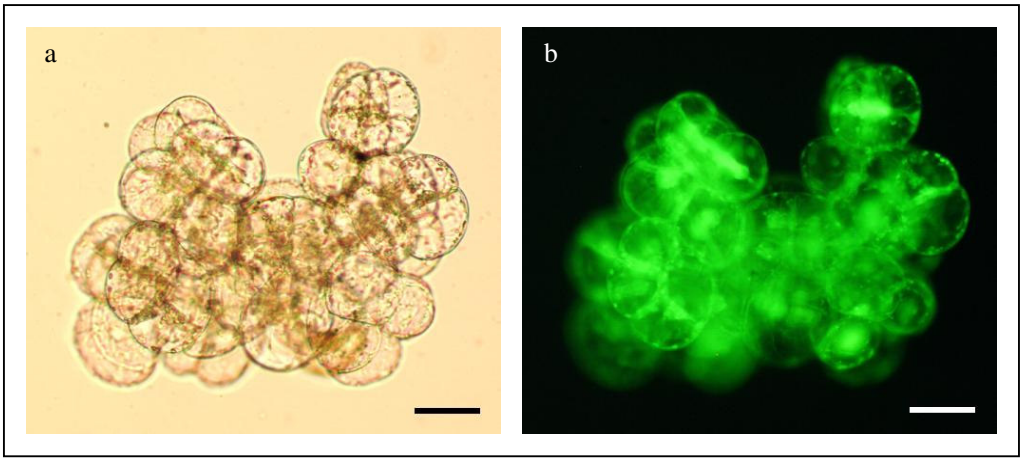


Figure 4.8. Callus proliferation in the form of small aggregates in genotype B3-2853.



a) Cells observed under the microscope with bright field illumination.

b) Same as a), observed under microscope with UV lamp using FDA staining.

Scale bars = 50 µm.

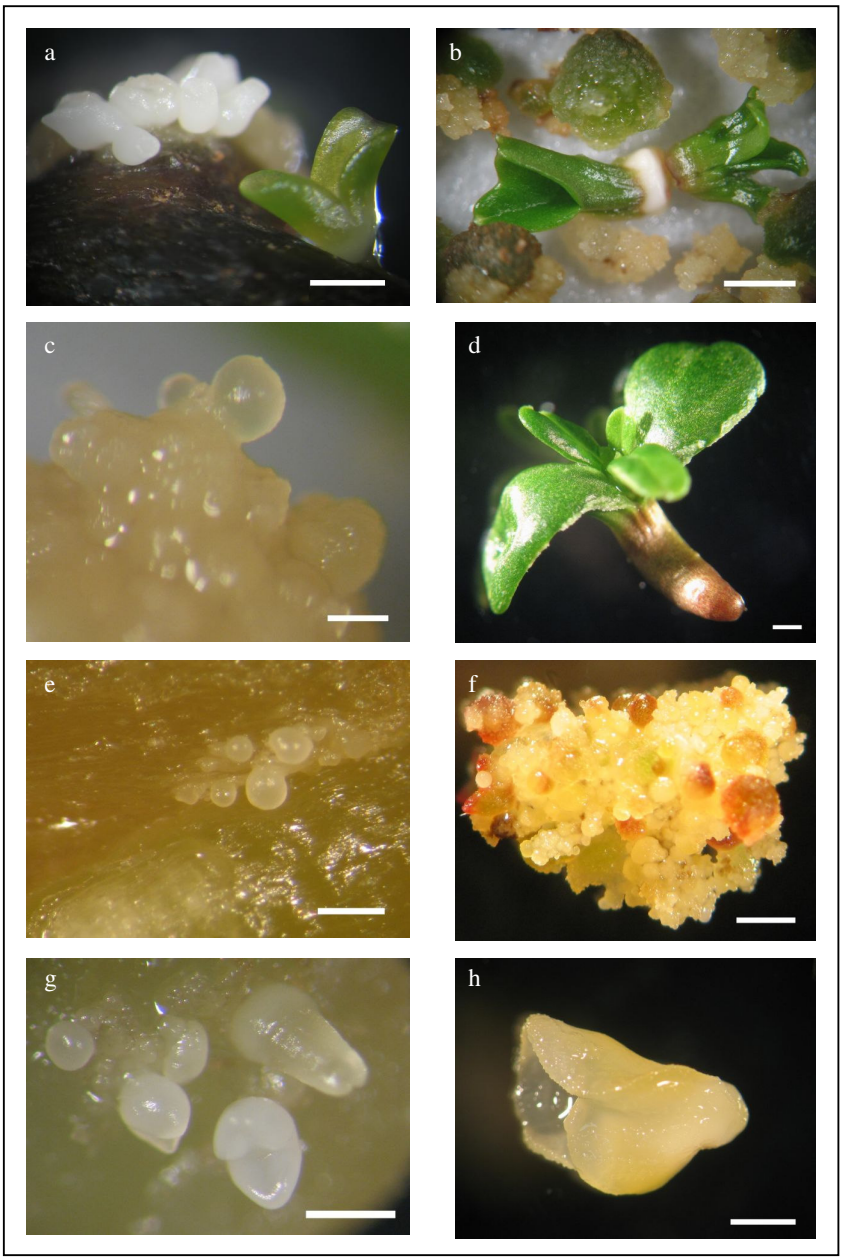
4.3.2 Somatic embryogenesis

Direct somatic embryogenesis was first observed on the radicle of a zygotic embryo (S3-172) after 96 d of culture on MS medium with 0.01 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l GA_3 (Figure 4.9a). During several subcultures of this material for more than one year, recurrent proliferation of somatic embryos was observed on semi-differentiated tissue as well as the production of friable white embryogenic callus on the same medium (Figure 4.9b, c). Maturation of somatic embryos was rarely observed as a green embryo over 5 mm long with a radicle zone and cotyledons (Figure 4.9d), the latter often being abnormal (Figure 4.9b). Green somatic embryos cultured in liquid media remained alive and produced some abnormal leaf primordial, but further development was not observed. Shoots developed from somatic embryos on semi-solid WP medium with 0.1 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l GA_3. Rooting of mature embryos of this genotype sometimes occurred after 6 months on WP medium containing 2 g/l AC and lacking PGRs (Figure 4.10a).

Embryogenic friable callus of genotype S3-172 cultured at 18 or 25°C and in the presence or absence of PVP-360,000 and PGRs in the medium showed growth and chlorophyll production regardless of the temperature or the media used. In some cases, callus developed a brown colour, possibly because of the production of phenolics. This browning seemed to be more frequent at 25 °C on medium without PGRs than with PGRs (Chi square test, P=0.0417), and at 18 °C on medium lacking PVP (Chi square test, P=0.003).

The experiment to induce somatic embryogenesis from zygotic embryos showed callus development, especially when 2,4-D was present for long time (7 months) in the medium (Table 4.4). Cultured endosperms showed no response. Some explants were lost due to contamination, while those in the light tended to develop chlorophyll. One explant cultured on MS medium with 0.1 mg/l 2iP and 0.01 mg/l NAA in the dark produced somatic embryos from the radicle zone after 7 months of culture. This second case of somatic embryogenesis from a zygotic embryo produced embryogenic callus as well as green embryos. One of these mature embryos rooted spontaneously after 2 months on WP medium with 2 g/l AC, but lacking PGRs (Figure 4.10b).

Figure 4.9. Development of somatic embryos of *Gomortega keule*.



a) Direct somatic embryogenesis on a zygotic embryo, genotype S3-172.

b) Secondary somatic embryos, genotype S3-172.

c) Initial stages of somatic embryogenesis, genotype S3-172.

d) Conversion of a somatic embryo, genotype S3-172.

e) Initial stages of somatic embryogenesis, genotype S3-112.

f) Embryogenic friable callus, genotype S3-112.

g) Early stages of somatic embryogenesis, genotype S3-112.

h) Somatic embryo with abnormal morphology, genotype S3-112.

Scale bars = 1 mm (a, b, d, f and h), 250 µm (c and e) and 500 µm (g).

Figure 4.10. Rooted somatic embryos of *Gomortega keule*.

Somatic embryos of genotype S3-172 (a) and S5-230 (b) after 6 (a) and 2 (b) months of culture on WP medium with 2 g/l AC without PGRs.
Scale bars = 10 mm (a) and 4 mm (b).

Using compact calli from liquid cultures of genotype S3-112, somatic embryos developed on MS medium with 0.01 mg/l NAA, 1.0 mg/l BAP, 0.1 mg/l GA_3 and 10 g/l PVP-360,000 after 74 d of culture (Figure 4.9e, g). This was noteworthy because somatic embryos arose from undifferentiated tissues (compact callus) and not directly from a zygotic embryo. Proliferation of friable embryogenic callus was observed on semi-solid medium (Figure 4.9f) and generally embryos grew with morphological abnormalities (Figure 4.9h). When small aggregates from liquid cultures were placed on semi-solid medium, they also proliferated giving friable embryogenic callus.

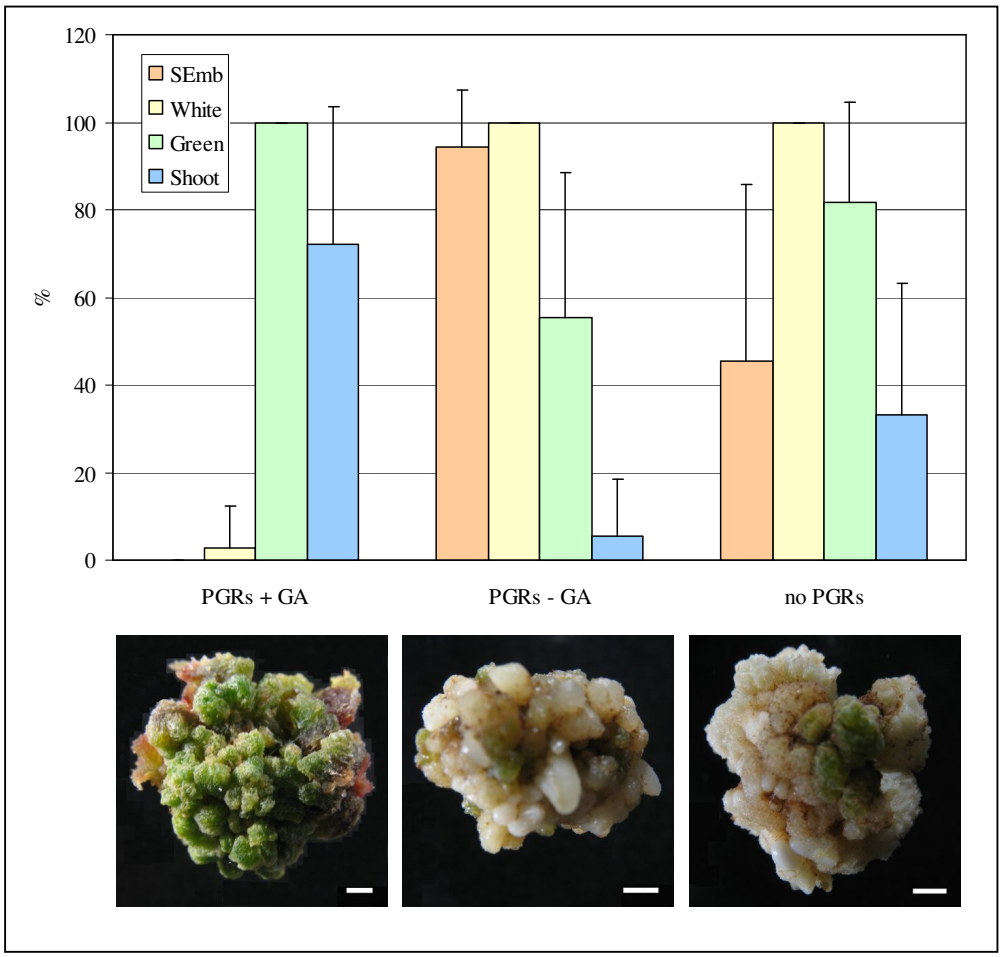
Table 4.4. Responses observed in zygotic embryos after 7 months of culture.

Treatment number	Number of embryos inoculated	Number of embryos evaluated	Embryos with callus		Embryos with shoots	
			Number	%	Number	%
1	12	3	0	0.0	1	33.3
2	28	17	1	5.9	3	17.6
3	30	20	2	10.0	11	55.0
4	30	19	4	21.1	1	5.3
5	30	24	9	37.5	1	4.2
6	27	22	22	100.0	1	4.5
7	27	17	4	23.5	2	11.8

Compact embryogenic callus of genotype S3-112 incubated for 90 d on semi-solid MS medium did not produce somatic embryos in the presence of GA_3 (Figure 4.11), but showed a greater frequency of production of somatic embryos when the medium had 0.01 mg/l NAA and 1.0 mg/l BAP than on medium with no PGRs (Mann-Whitney test, P=0.003). Green callus developed in all explants in the presence of GA_3, while without GA_3 green callus was observed at higher frequency when the medium had no PGRs (t-test, P=0.037). The production of semi-differentiated shoot-like tissues was stimulated in the presence of 0.1 mg/l GA_3 (Mann-Whitney test, P<0.0001) and had a lower frequency on medium with 0.01 mg/l NAA and 1.0 mg/l BAP than on medium with no PGRs (Mann-Whitney test, P=0.0149).

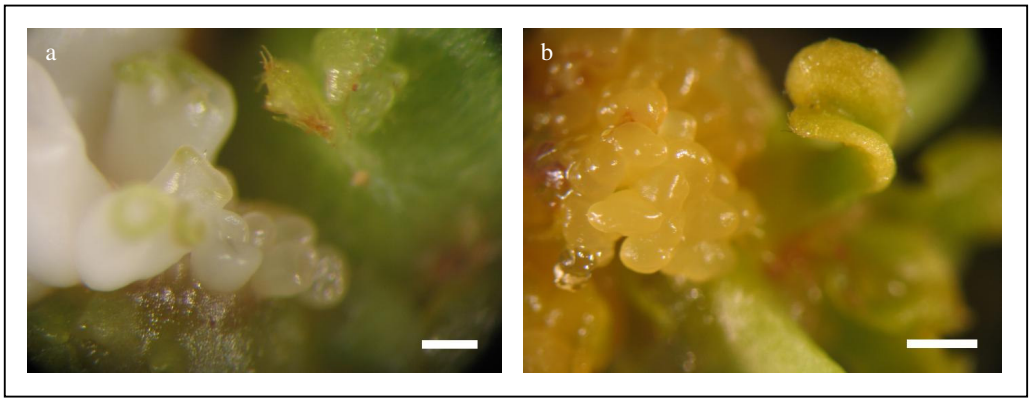
The second experiment, aimed to induce somatic embryogenesis in shoots of genotypes S3-60 and S3-104, produced callus after 5 months of culture, but somatic embryos were not observed. However, in the third experiment, somatic embryos occurred in shoots of genotype S4-A3 after 6.5 months (196 d), while on the shoots of genotype S3-104, compact embryogenic-like structures with a light colour developed (Figure 4.12, Table 4.5). When comparing the responses for the different concentrations of PGRs, no significant differences were found (Chi-square tests) within genotype.

Figure 4.11. Response of compact callus after 90 d of culture on MS medium.



Frequency (%) of development of somatic embryos (SEmb), white callus (White), green callus (Green) and semi-differentiated tissue (Shoot) on medium with 0 or 0.1 mg/l GA_3 (+ or -GA) and with 0.01 mg/l NAA and 1.0 mg/l BAP (PGRs), or without PGRs. Scale bars = 2 mm.

Figure 4.12. Somatic embryos developed on shoots of *Gomortega keule*.



a) Shoots of genotype S4-A3 cultured on MS medium with 0.1 mg/l 2,4-D and 0.1 mg/l 2iP for 196 d.

b) Embryogenic-like structures of S3-104 cultured on MS medium with 1.0 mg/l 2,4-D and 0.1 mg/l 2iP for 196 d.

Scale bars = 0.3 and 1 mm.

Table 4.5. Number of shoots with somatic embryos (genotype S4-A3) or embryogenic tissue (genotype S3-104).

PGR concentration (mg/l)		S4-A3		S3-104	
2,4-D	2iP	induced	total [1]	Induced	total
0.1	1.0	3	12	0	3
1.0	1.0	5	12	6	12
0.1	0.1	4	12	1	8
1.0	0.1	2	12	6	12

[1] total number of shoots evaluated after 196 d.

4.4 Discussion

4.4.1 Cultures in liquid media

There seemed to be a strong dependence on genotype to successfully initiate a culture in liquid medium. For most genotypes, when initiating cultures, cells seemed to be highly vacuolated, large in size and elongated or irregularly shaped (Figure 4.1f), which are typical

signs of non-embryogenic cells (Jayasankar *et al*. 1999). Probably these free cells detached from the explants used to inoculate the cultures.

The evaluation of growth using compact callus of genotype S3-112 in liquid MS medium with concentrations of NAA (0 and 0.01 mg/l) and 2iP (0 and 0.1 mg/l) showed an effect of 2iP, while the experiment evaluating the effect of NAA or 2,4-D showed no difference for the lowest concentration, or the absence of PGRs (Figure 4.6). The growth rate for the treatment lacking PGRs was 0.09 g/d, although further experiments are needed to confirm that the medium without PGRs sustains a higher growth than medium containing PGRs. Compact callus also proliferated in liquid medium from somatic embryos of genotype S3-172. For both genotypes, large callus masses showed browning and lack of new tissues in active growth (light coloured), which was interpreted as an ageing symptom. Those masses needed to be cut into smaller explants to re-activate the cultures.

Small aggregates of genotype S3-112 showed optimum proliferation in MS liquid medium with 0.01 mg/l NAA and 0.1 mg/l 2iP. Liquid cultures of small aggregates were regarded as easier to manipulate than cultures of compact callus. Interestingly, callus derived from a bud of a field-collected shoot (genotype B3-2853) proliferated in liquid medium and after 18 months of culture produced small aggregates. The cells showed non-embryogenic characteristics under the microscope (Figure 4.8). When incubated on semi-solid MS medium with 0.01 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/l GA_3, the aggregates grew but organogenesis was not observed. This represented the only sustained proliferation of tissues from field-collected shoots obtained in the present study. Importantly, future experiments inducing somatic embryogenesis on proliferating callus might open an alternative path to micropropagation of selected mature trees of *G. keule*.

Cultures of *G. keule* in liquid medium may enable the development of methods for mass propagation, especially if the cultured material shows somatic embryogenesis. Liquid cultures of somatic embryos have been developed for the endangered medicinal woody plant *Eleutherococcus senticosus* (Choi *et al*. 2002). Cultures in liquid medium may also be relevant for secondary metabolite production, as it has been explored for the medicinal tree *Eucommia ulmoides* (Miyanaga *et al.* 2004). This area will remain restricted until further research on the chemical components of *G. keule* and their activities is carried out.

4.4.2 Somatic embryogenesis

Spontaneous somatic embryogenesis occurring directly on zygotic embryos of *G. keule* was observed as a rare phenomenon at a low frequency (2 in *ca.* 560 embryos cultured, 0.35%). Somatic embryogenesis has been reported in mature zygotic embryos of the endangered tree *Ocotea catharinensis* (Moura-Costa *et al*. 1993). In micropropagated shoots of *G. keule*, somatic embryogenesis was observed after prolonged exposure to 2,4-D, and was not recurrent. Further research is needed to improve induction conditions, especially using shoots of selected genotypes.

Plants obtained from rooted somatic embryos of genotype S3-172 seemed less vigorous than those derived from rooted normal shoots. One of the former plants survived 5 months after being transferred to the glasshouse. Probably, the size of the regenerated plant prior to acclimatization is an important factor for its survival in the glasshouse, since it was observed that larger plants (originated from micropropagated shoots, Chapter 3) survived and started to grow during the acclimatization process. In contrast, small plants generally died.

Somatic embryos of *G. keule* grew with morphological abnormalities. This fact is considered to be common in woody species (Yoeup and Chakrabarty 2003). Using compact callus grown in liquid medium, the optimum conditions to allow somatic embryo development were on semi-solid MS medium with 0.01 mg/l NAA, 1.0 mg/l BAP and lacking GA_3. However, proliferation of embryogenic tissues of genotype S3-112 seemed to be optimum in liquid medium. Regarding the time of incubation and the need to manipulate compact callus (cut and transference using forceps), the culture of small aggregates in liquid medium was regarded as preferable. This procedure would allow the mass production of tissues capable of regeneration into whole plants.

The first experiment to induce somatic embryogenesis in shoots included 7 d on semi-solid MS medium with 2,4-D (0.1 or 1.0 mg/l) and 1.0 mg/l 2iP, followed by incubation on MS medium with 0.01 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/l GA_3. This experiment produced callus after 5 months, but somatic embryos were not observed. However, the occurrence of somatic embryogenesis on a shoot after 8 months on MS medium with 0.1 mg/l 2,4-D and 0.1 mg/l 2iP, indicated the possibility of inducing somatic embryos with prolonged exposure to PGRs at greater concentrations. In fact, somatic embryogenesis was observed on shoots of genotype S4-A3 after 6.5 months on semi-solid MS medium supplemented with 2,4-D and 2iP, regardless of the concentration of these PGRs. Secondary somatic embryogenesis was not

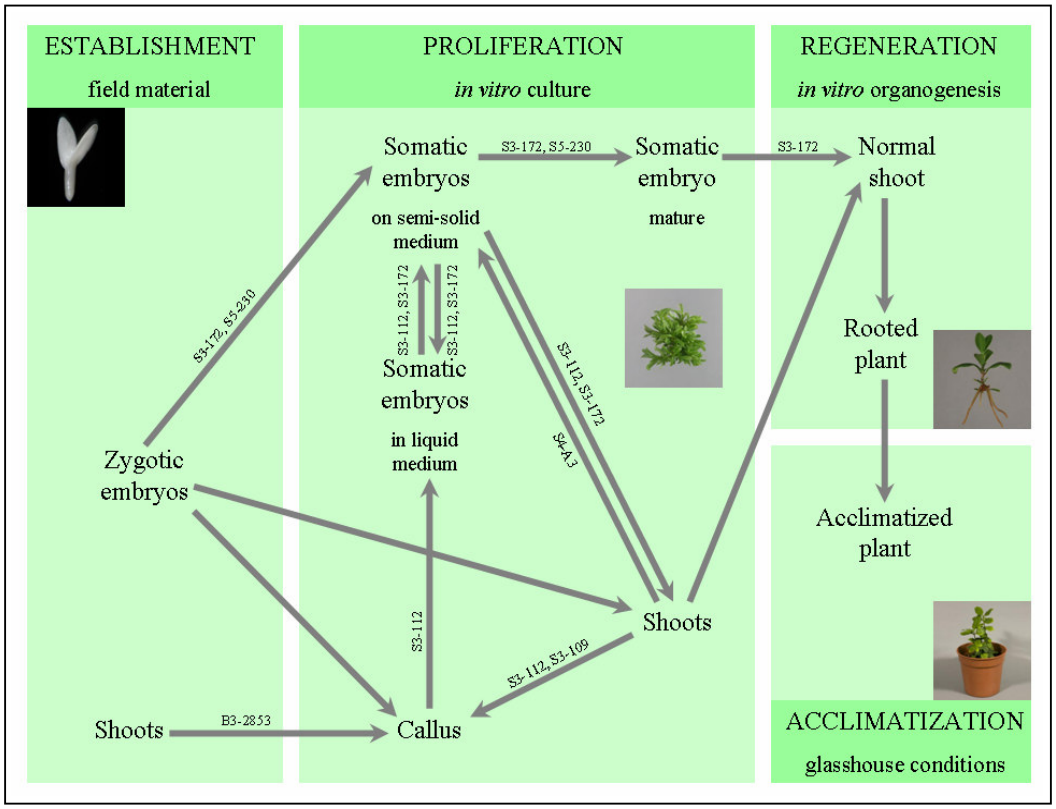
observed after incubation on MS medium with 0.01 mg/l NAA, 1.0 mg.l BAP and 0.1 mg/l GA_3, which was previously shown to allow repetitive somatic embryogenesis in genotypes S3-112 and S3-172. Thus, secondary somatic embryogenesis in shoots of genotype S4-A3 may require the permanent presence of 2,4-D and 2iP. The structures observed on shoots of genotype S3-104 may correspond to embryogenic tissues or abnormal embryos. Further research is needed, applying permanent exposure to 2,4-D and 2iP to shoots of selected genotypes.

The fact that direct, indirect and repetitive embryogenesis were observed in 5 different genotypes, indicates that the process of somatic embryogenesis in *G. keule* may not be too rare and that explants of different types can produce somatic embryos. Somatic embryogenesis is known to be a process highly dependent on species and genotypes (Kumar *et al*. 2006; Pollastri 2008; Samson *et al*. 2006; Singh *et al*. 2003; Valladares *et al*. 2006), which seemed to be the case for *G. keule*.

Somatic embryogenesis in *G. keule* represents a base for further work on mass propagation and cryopreservation. Specifically, improved induction of somatic embryogenesis from non-seed tissues, and more importantly from adult elite trees, along with the maturation and conversion of somatic embryos, would be the next targets for research in this area.

The development of protocols for cultures in liquid medium and for the induction of somatic embryogenesis is important for the micropropagation of *G. keule*. These findings, including the results reported in Chapters 2 and 3, permit tracing of the possible stages, paths and procedures to regenerate plants from field-collected material (Figure 4.13), and will permit the production of plants ready for its establishment in the field, for commercial or conservation purposes.

Figure 4.13. Overview of micropropagation stages for *Gomortega keule*.



Initial and resulting structures are linked by arrows. The genotype(s) where a given response was observed is indicated on the arrow, otherwise several genotypes showed the response.

4.5 Conclusions

Cultures of compact callus of genotype S3-112 showed optimum proliferation in liquid MS medium with 20 g/l sucrose, 0.01 mg/l NAA and 0.1 mg/l 2iP. Small embryogenic aggregates also proliferated in liquid medium under those conditions. Somatic embryogenesis was observed from compact calli and from small aggregates when they were transferred onto MS semi-solid medium with 0.01 mg/l NAA and 1.0 mg/l BAP.

Recurrent somatic embryogenesis was observed directly from the radicle zone of zygotic embryos in two cases, leading to the proliferation of friable embryogenic callus on MS semi-solid medium with 0.01 mg/l NAA and 1.0 mg/l BAP.

Using micropropagated shoots, somatic embryogenesis was induced after 6.5 months on semi-solid MS medium supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l 2iP.

Cultures of embryogenic material of *G. keule* in liquid medium may allow the propagation of selected genotypes of this tree on a mass scale.

Chapter 5

Cryopreservation and cytology of *Gomortega keule*

5.1 Introduction

5.1.1 Cryopreservation

Cryopreservation is a technique to maintain living tissues for very long periods of time at ultra low temperatures (-196 ºC) immersed in liquid nitrogen (LN). A first problem for survival of plant tissues is the physical and biochemical injuries caused by ice formed either during the freezing process or the thawing stages. To avoid this, dehydration of tissues is necessary to allow the intra- and extra-cellular solutions to become highly concentrated, resulting in a metastable amorphous glassy state (vitrified) at low temperatures, minimizing the formation of ice crystals and concomitant damage to the cells. A second problem is the cold tolerance of plant tissues, especially for tropical species. The technique was first applied to plants at the end of the 1960s. There are two main groups of cryopreservation methods in plants, namely, classical techniques and vitrification-based protocols (Benson *et al*. 2006; Gonzalez-Arnao *et al*. 2008).

The classical, traditional or controlled rate techniques use a pretreatment with cryoprotective solutions and gradual cooling over a period of time, allowing the slow formation of ice in the extracellular solution, removing water from the interior of the cells. The controlled cooling process to -40°C is achieved using expensive equipment after which the samples are immersed rapidly in LN, which produces crystallization of the remaining water, or vitrification of intracellular solutions. During cooling, extracellular crystallization process is the key step, while during thawing of samples a rapid warming rate is crucial. The traditional techniques have proven to be useful mainly in temperate species, especially for small explants with undifferentiated cells such as meristems, somatic embryos and cell suspensions

(Gonzalez-Arnao *et al*. 2008; González-Benito *et al*. 2004; Engelmann 2004). The regeneration of plants occurs usually by organogenesis from callus, especially in tropical species, which is undesirable (Gonzalez-Arnao *et al*. 2008), as it is considered to be a possible source of genetic variation.

The vitrification-based techniques were developed from the late 1980s and they use a dehydration process, either by exposure to highly concentrated cryoprotective solutions, or sometimes by physical drying conditions. Vitrification is usually achieved with a concentrated solution (PVS2) containing sucrose, glycerol, ethylene-glycol and dimethyl sulfoxide (DMSO), as developed by Sakai *et al*. (1990), which is the most frequently used vitrification solution (Benson 2008). Because the vitrification solution can be toxic or cause osmotic damage to plant tissues, previous steps to increase survival are performed with 'loading solution' as well as 'unloading solution' after cryopreservation. Additional measures to prevent osmotic shock can be attempted with preculture treatments, preloading solutions, application of PVS2 on ice and stepwise increasing concentrations of PVS2 (Benson 2008). Cooling is applied by direct immersion of the sample in LN, allowing the formation of a metastable glass in the plant tissues. A number of different modifications of the vitrification technique have been developed incorporating preculture, encapsulation and air drying of tissues. The critical step is dehydration and not freezing as in classical protocols (Gonzalez-Arnao *et al*. 2008; González-Benito *et al*. 2004; Engelmann 2004). These modern techniques have been applied to cryopreserve more complex tissues comprising differentiated cells, such as shoot tips, allowing recovery without callus formation and thus avoiding genetic instability. Another advantage of modern techniques is that they can be applied to several temperate and tropical species (Gonzalez-Arnao *et al*. 2008), including shoot tips of trees such as *Citrus* (Al-Ababneh *et al*. 2002). Also, they do not require the use of controlled freezing equipment (Engelmann 2004).

In trees, cryopreservation has been achieved using different sources of material such as zygotic embryos, embryogenic cultures, shoot tips and dormant buds. Shoot tips of several woody plants including *Malus*, *Morus*, *Diospyros*, *Populus*, *Pyrus* and *Vitis* have been cryopreserved, an even some tropical herbaceous plants (Sakai *et al*. 2002). The cryopreservation of dormant winter buds is also possible (Benson *et al*. 2006). Using shoot tips and also embryogenic cultures or somatic embryos, tree species have been successfully cryopreserved, particularly in the genus *Citrus*, *Populus* and *Prunus* (Sakai and Engelmann 2007). Generally, temperate trees can be cryopreserved using different types of explants, but the survival of material after storage in LN for tropical species have been achieved mainly for embryogenic cultures. Temperate species bear cold hardiness, acclimatization and dormancy

characteristics, which confer to them advantages for cryopreservation, but tropical and warm temperate species are sensitive to cold and dehydration (Benson *et al*. 2006). The genus *Citrus* can be considered an exception amongst tropical species, where cryopreservation of shoot tips has been reported (Al-Ababneh *et al*. 2002). In olive tree, shoot tips survived cryopreservation and produced callus, but died after 8 - 10 weeks (Lynch *et al*. 2007). Amongst vegetatively propagated species of commercial importance, germplasm banks in different countries currently include in their collections cryostored material of *Malus*, *Morus*, *Vitis*, *Pyrus*, *Musa*, *Manihot* and *Fragaria* (Engelmann 2004).

5.1.2 Cytology

In vitro culture of plant materials is subjected to mutagenesis, especially when the processes involve an undifferentiated callus phase. The variation includes chromosomal rearrangements, single-gene mutants (mostly recessive) and DNA methylation changes in regenerated plants and their progeny. Regenerated plants frequently suffer chromosome breakage, which results in chromosome aberrations. This has been well characterized in cereals and also observed in other species (Phillips *et al*. 1994).

Cytological studies, involving the number and morphology of the chromosomes, have been employed to assess the genetic uniformity of material propagated *in vitro*, together with morphological identification of plants, isozyme electrophoresis and, more recently, with DNA-based molecular techniques (Rani and Raina 2000). Chromosomes of *in vitro* regenerated plants of the medicinal herb *Swertia chirata* showed no variation in number (Chaudhuri *et al*. 2007). A similar situation was found by Lattoo *et al*. (2005) in *Chlorophytum arundinaceum* for regenerated and mother plants and in the endangered woody species *Lippia filifolia* (Peixoto *et al*. 2006). Using a multidisciplinary approach employing morphological evaluations, molecular markers (RAPD) and cytological examination of *Betula pendula* plants regenerated after short or long-term tissue culture, and with or without cryopreservation, Ryynänen and Aronen (2005) reported no genetic or phenotypic changes, concluding that micropropagation can be considered as reliable to conserve the genetic characteristics of the material. In *Hypericum perforatum*, plants recovered from cryopreserved meristems showed no cytological changes or differences of hypericin content, but minor alterations in non-coding DNA sequences were detected with variable number tandem repeat (VNTR) in comparison to unfrozen controls (Urbanová *et al*. 2006). In cultured callus of *Solanum tuberosum*, Vargas *et al*. (2008) found polyploid cells but, interestingly, somatic

embryos and regenerated plants from that material were euploid, which they attributed to the selection of cells during the culture process. They also reported very low frequency of genetic variation as detected by RAPD, with no effect on phenotype. Changes in the number of chromosomes have been observed for several cereals propagated *in vitro*, as well as for some conifers (Rani and Raina 2000). Cytological analysis has also been applied to assess the genetic uniformity of shoot tips after cryostorage, this being shown to be stable for many species (Harding 2004).

In *G. keule*, cytological assessments have never been made of cultured material or regenerated plants. Goldblatt (1976) reported a chromosome number of $2n = 42$. More recently, Baeza *et al*. (2001) observed the same number, but Oginuma and Tobe (2006) discussed the need for confirmation, since 42 chromosomes represent a sufficiently large number to miscount. The observation of chromosomes in *G. keule* will be of interest to confirm previous reports on the species number and to provide evidence of genetic stability of micropropagated and cryostored material.

The objectives of the chapter were:
1) To explore cryopreservation for *in vitro* cultured material.
2) To determine the ploidy of regenerated plants and *in vitro* cultured explants of *G. keule*.

5.2 Materials and Methods

5.2.1 Plant materials

Cryopreservation experiments were performed using shoot tips of genotypes S3-60, S3-104 and S3-109 cultured on WP medium supplemented with 0.1 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l GA_3 (Chapter 3). Embryogenic callus (small aggregates) of genotypes S3-112 cultured in MS liquid medium (with 0.01 mg/l NAA and 0.1 mg/l 2iP) and friable embryogenic callus of genotype S3-172 cultured on MS semi-solid medium (Chapter 4), were also used.

Embryogenic callus of genotypes S3-112 and S3-172, and non-embryogenic callus of genotype B3-2853 (Chapter 4) were exploited for cytological analysis. Additionally, normal shoots *in vitro* were induced to root with 7 d incubation on WP medium containing 20 mg/l

IBA, followed by culture on WP medium with 2 g/l AC. The tips of the resulting roots were analysed along with those of acclimatized plants (Chapter 3).

5.2.2 Cryopreservation procedures

As *G. keule* is a tree bearing evergreen leaves from a mid-latitude region with mild winters, the species can be considered as being subtropical for cryopreservation purposes. This, together with the easier procedures associated with modern techniques and the availability of shoot tips from *in vitro* cultures, resulted in the choice of the techniques and explants mentioned to initiate cryopreservation experiments.

The general procedure followed involved preculture in sucrose-enriched (0.3 M) WP medium, incubation in loading solution at room temperature, incubation in vitrification solution (PVS2), storage in LN, thawing in a warm water bath for 2 min, incubation in unloading solution (1.2 M sucrose) at room temperature and on recovery medium. Before plunging the explants into LN, they were placed in 2 ml cryovials (Sarstedt Ltd., Leicester, UK), the cryovials were capped and set in an aluminium cryocane. For shoot tips, the recovery medium was semi-solid WP medium supplemented with 0.1 mg/l NAA, 1.0 mg/l BAP and 0.5 mg/l GA_3 at 18°C, while for embryogenic callus it corresponded to semi-solid MS medium but with 30 g/l sucrose, 0.01 mg/l NAA, 1.0 mg/l BAP and 0.1 mg/l GA_3 at 25ºC. In order to improve the response to cryopreservation treatments, modifications to the protocols were introduced using different preculture times, types of explants, times of exposure to vitrification solution, type of vitrification solution, thawing temperatures, sucrose concentrations in the unloading solution, light intensity and sucrose concentration in the medium during the recovery stage. Controlled rate freezing and encapsulation-vitrification were also used. Explants were incubated in 1.5 ml of each solution, except for the freezing step that was performed using 0.5 ml of fresh vitrification solution. The preparation of media and solutions is detailed in Appendix 9.18. The procedures applied are summarized in Table 5.1.

5.2.2.1 Cryopreservation of shoot tips

Before the preculture, shoot tips from actively proliferating genotypes were dissected and the leaves trimmed to obtain the explants each less than 5 mm long with 1 or 2 apices. After incubation in unloading solution, the shoot tips were blotted on sterile filter paper and incubated on recovery medium. For evaluation, green shoot tips were counted.

Table 5.1. Cryopreservation procedures applied to explants of *Gomortega keule*.

Explant	Experiment	Genotype	Preculture (time) [1]	Loading solution (time)	PVS2 (time, temperature)	Thawing (temperature) [2]	Unloading solution (time)	Recovery medium (time) [3]
Shoot tips	First experiment	S3-60	10 d	30 min	20 min, room temperature	35ºC	10 min	2 d, 15 d
	Sucrose in unloading solution	S3-104	9 d	30 min	20 min, room temperature	- [4]	20 min [5]	2 d, 22 d
	Incubation in PVS2	S3-104	9 d	30 min	20, 40, 60 80, 100 min, room temperature	- [4]	20 min	1 d, 20 d
	Incubation in PVS2	S3-104	18 d	30 min	5, 10, 15, 20, 25 min, room temperature	- [4]	30 min	2 d
	Incubation in PVS2	S3-109	7 d	30 min	20, 40, 60, 80, 100 min, on ice	35ºC [6]	20 min	21 d
	Temperature of water bath	S3-109	9 d	30 min	30 min, on ice	25, 30 , 35ºC	20 min	1 d, 4 d
	Preculture treatments	S3-109	21 d [7]	30 min	30 min, on ice	35ºC	20 min	2 d, 6 d
	Slow-cooling technique [8]	S3-104	11 d	30 min	60 min, on ice	35ºC	20 min	28 d
	Vitrification solution	S3-109	15 d	30 min	80 min, on ice [9]	35ºC	20 min	3 d, 28 d [10]
	First stepwise PVS2	S3-104	37 d	30 min	60 min, on ice [11]	35ºC	20 min	4 d, 28 d [10]
	Second stepwise PVS2	S3-109	49 d	30 min	60 min, on ice [11]	35ºC	20 min	4 d, 28 d [10]

Table 5.1. Cryopreservation procedures applied to explants of *Gomortega keule* (continued).

Explant	Experiment	Genotype	Preculture (time) [1]	Loading solution (time)	PVS2 (time, temperature)	Thawing (temperature) [2]	Unloading solution (time)	Recovery medium (time) [3]
Embryogenic callus	First experiment	S3-172	no	30 min	20 min, room temperature	35ºC	20 min	47 d
	Slow-cooling technique [8]	S3-172	no	15 min	10 min, on ice	35ºC	10 min	47 d
	One-step freezing	S3-172	no	15 min	10 min, on ice	35ºC	20 min	3 d, 32 d [12]
	Encapsulation-vitrification	S3-112	no	20 min	10 min, on ice	35ºC	20 min	1 d, 28 d [12]

[1] Preculture on sucrose-enriched (0.3 M) WP medium.

[2] Thawing lasted for 2 min after 1 h storage in liquid nitrogen.

[3] Times indicated corresponded to transfer to fresh recovery medium, while the last number was the time at evaluation.

[4] Treatment not subjected to cryostorage and thawing.

[5] Sucrose concentrations tested in the unloading solution were 0.2, 0.4, 0.8 and 1.2 M.

[6] Treatment not subjected to cryostorage.

[7] Treatments also included one additional day on medium with 0.7 M sucrose, a second day with 1.0 M sucrose, and the last 2 days (0.7 and 1.0 M sucrose) at 5ºC in the dark.

[8] After incubation in PVS2, explants were maintained 10 min at 0°C, 40 min cooling at a rate of -1°C/min, 30 min at -40°C and 1 h in LN.

[9] PVS2 and VSL were tested.

[10] Incubated in the dark on sucrose-enriched (0.3 M) WP medium without PGRs, then transferred to recovery medium in the light.

[11] Shoot tips were placed in 1 ml 50 % PVS2 solution (loading solution:PVS2, 1:1) for 20 min, then into 1 ml PVS2 for 20 min, and placed in fresh 0.5 ml PVS2 for 20 min.

[12] Incubated in the dark, then in the light.

In the first experiment, sterile Pasteur pipettes were used to remove and add solutions to autoclaved 2 ml cryovials (Sarstedt Ltd., Leicester, UK) in the laminar flow cabinet, and sterile forceps to transfer the shoot tips to a new vial. Later, however, micropipettes with sterile plastic tips were used to facilitate handling. Controls were taken after preculture on sucrose-enriched medium (7 shoot tips), incubation in loading solution (10 shoot tips) and incubation in PVS2 solution (12 shoot tips). Before plunging the cryovials in LN, PVS2 was changed for new PVS2.

The effect of sucrose concentration (0.2, 0.4, 0.8 and 1.2 M) in the unloading solution was evaluated using 2 vials each with 10 shoot tips and 1 with 11 tips. A control (1 vial with 11 shoot tips) was not stored in LN.

In order to assess the effect of incubation time in PVS2, 2 experiments were performed, incubating in PVS2 for 20, 40, 60, 80 or 100 min. For one experiment, incubation in PVS2 at room temperature was performed with genotype S3-104 using 1 vial per treatment, each with 9, 11, 12, 13 and 14 shoot tips. With the same genotype, shorter times (5, 10, 15, 20 and 25 min) were tested using 3 vials with 13 shoot tips and 2 with 14 tips (1 vial per treatment). A control was taken after preculture (1 vial with 10 shoot tips). An additional experiment to test incubation in PVS2 for 20, 40, 60, 80 or 100 min was carried out using 3 vials per treatment, each with 5 shoot tips.

The temperature of the water bath for the thawing stage was evaluated at 25°C (2 vials), 30°C (2) and 35°C (1 vial). All vials each had 5 shoot tips each. Preculture treatments were applied with the objective to evaluate their effect on survival after cryopreservation, using 1 cryovial per treatment with 6 shoot tips in each cryovial. To evaluate the slow-cooling technique, 4 vials (8 shoot tips each) were placed in a controlled rate freezer (Planer Kryo 10 Series II) and the program run was 10 min at 0°C, 40 min cooling at a rate of -1°C/min and 30 min at -40°C. The vials were quickly plunged into LN.

In experiments to compare 2 different vitrification solutions, shoot tips in 3 vials were treated with PVS2, and 1 vial with VSL. After storage in LN, the shoot tips were incubated in 1.5 ml of unloading solution and the solution changed for fresh unloading solution after 10 and 15 min. The shoot tips were incubated on sucrose-enriched (0.3 M) WP medium for 3 d and then placed on recovery medium. As a control, 1 cryovial in each treatment followed the whole procedure but was not stored in LN.

Cryopreservation was also assessed using a stepwise increase in PVS2 concentration with shoot tips dissected to obtain explants of *ca.* 3 mm in size. Shoot tips were incubated in 1 ml 50 % PVS2 solution (loading solution:PVS2, 1:1), transferred to 1 ml PVS2, and placed in fresh 0.5 ml PVS2. Controls of 7 shoot tips for genotype S3-104 and 9 for S3-109 were not plunged in LN but followed the rest of the procedure. After thawing, the shoot tips were placed in 1.5 ml of unloading solution, this solution being changed for fresh unloading solution after 10 and 15 min. The shoot tips were placed onto sucrose-enriched (0.3 M) WP medium and transferred onto recovery medium.

5.2.2.2 Cryopreservation of embryogenic callus

A first experiment using friable embryogenic callus was performed by placing the tissue in 4 vials. A control vial was not stored in LN but proceeded through the protocol. After the treatment with the unloading solution, the embryogenic aggregates were taken with forceps and spread on proliferation medium. Aggregates were observed by light microscopy using FDA staining. A protocol using the slow-cooling technique was performed, treating 3 vials with 0.5 ml PVS2 in a controlled rate freezer (same conditions as detailed in Section 5.2.2.1). Aggregates were observed by microscopy with FDA staining and later subcultured to new proliferation medium. A second experiment with direct immersion of 3 vials in LN was performed, with a control (1 vial) lacking LN storage. The unloading solution was changed for new unloading solution at 10 and 15 min.

The encapsulation-vitrification technique was performed using small embryogenic aggregates of genotype S3-112 cultured in liquid medium. The liquid culture was placed in a 50 ml plastic centrifuge tube and allowed to settle down, obtaining 3 ml of sedimented tissues. The supernatant was discarded and alginate solution was added, bringing the volume to 20 ml with gentle mixing. Using flame-sterilized scissors, micropipette plastic tips were cut in order to have a wide bore and drops of alginate solution with aggregates were delivered onto the MS liquid medium with calcium. The resulting beads were allowed to polymerize for 15 min with gentle inversion. The beads were blotted on sterile filter paper. Except for 1 control, 4 vials were plunged into LN. After thawing, PVS2 was changed for unloading solution, changing for fresh unloading solution at 10 and 15 min. The beads of 1 vial were transferred to MS liquid medium with 20 g/l sucrose, 0.01 mg/l NAA and 0.1 mg/l 2iP, with agitation in the dark. The beads of the remaining 3 vials were incubated on embryogenic proliferation medium and subcultured every 4 weeks.

5.2.3 Cytological observations

Root tips from acclimatized and *in vitro* cultured plants were excised and treated with a solution of 2 mM 8-hydroxyquinoline (Sigma) for 24 h at room temperature. Small embryogenic aggregates of genotype S3-112 and non-embryogenic callus of genotype B3-2853 cultured in liquid medium were incubated for 24 h with 2 mM 8-hydroxyquinoline. After that treatment, the material was placed in fixative (ethanol:acetic acid, 3:1) for at least 16 h. Hydrolysis was performed in 1 N (embryogenic aggregates) or 5 N (roots) hydrochloric acid at 65 °C for 20 min and the samples were washed 3 times in reverse-osmosis water. Each sample was placed on a glass slide and stained with 50 µl of 1% (v/v, in 0.45 M acetic acid) aceto-orcein (TAAB Laboratories Equipment Ltd, Aldermaston, UK). The slide was gently warmed and a glass cover was placed on the tissue. The sample was macerated by tapping on the glass cover using a pen.

Alternatively, after hydrolysis, the procedure described by Andras *et al*. (1999) was followed (preparation of solutions detailed in Appedix 9.19). The tissue was placed in 100 µl TE buffer and then in 200 µl of enzyme mixture in a 1.5 ml centrifuge tube, mixed by pipetting and incubated for 30 min at 37ºC. Reverse-osmosis water (400 µl) was added and the tube centrifuged for 1 min at 4000 rpm. The supernatant was discarded and the pellet resuspended in 600 µl water. The tube was centrifuged for 1 min at 4000 rpm, the supernatant discarded and the pellet resuspended in 10 µl water. Methanol-acetic solution (600 µl) was added and the tube centrifuged for 1 min at 4000 rpm. The supernatant was discarded and the pellet resuspended in 150 µl methanol-acetic solution. Drops of 8 µl were placed on glass slides and allowed to air dry. The samples were stained with 50 µl of aceto-orcein, warmed over a spirit lamp and glass cover slips applied.

Cells were observed and photographed by light microscopy using an oil immersion objective lens. From single cells, 1 - 11 photographs were taken to cover different depths of focus and prints were prepared to count chromosomes.

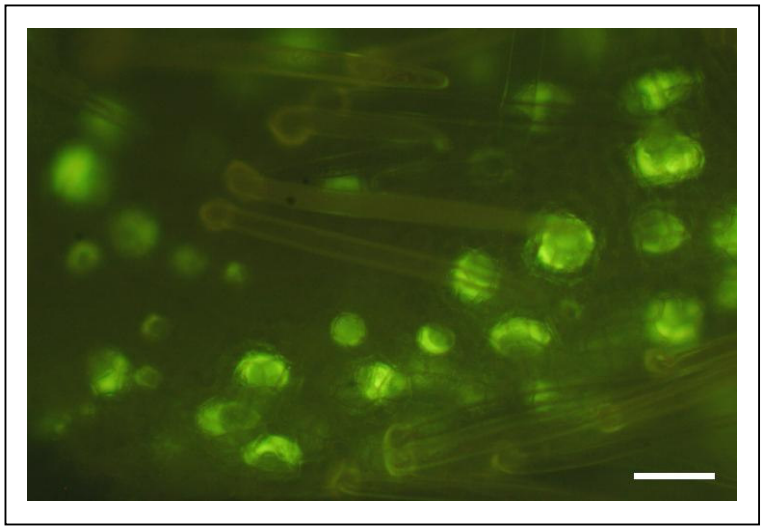
5.3 Results

5.3.1 Cryopreservation

5.3.1.1 Cryopreservation of shoot tips

Isolated cells in shoot tips were observed to be alive after 100 d of incubation from cryostorage in the vitrification solution experiment, involving treatment with PVS2 (Figure 5.1).

Figure 5.1. Shoot tip with living cells after cryopreservation.

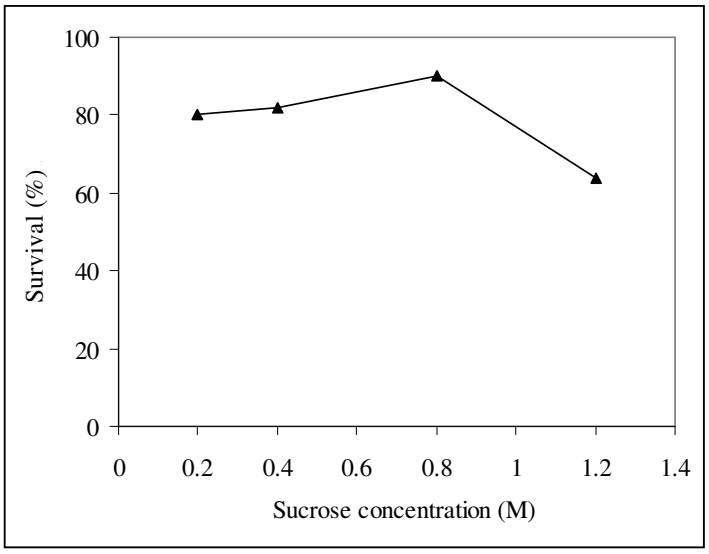


Scale bar = 50 μm.

In the first experiment, all 7 shoot tips from the first control (after preculture) and the 10 shoot tips from the second control (after incubation in loading solution) survived. From the third control (after incubation in PVS2), 6 tips survived (50%). All the 30 shoots stored in LN died.

Different sucrose concentrations in the unloading solution evaluated after 23 d (no LN treatment) showed no effect on shoot tip survival (Figure 5.2). The most common concentration (1.2 M sucrose) reported in the literature (Benson *et al*. 2006; Gonzalez-Arnao *et al*. 2008) was preferred for further experiments.

Figure 5.2. Survival of shoot tips treated with different sucrose concentrations in the unloading solution.



The experiments to assess the effect of exposure time to PVS2 showed a sharp effect on shoot tip survival. The first experiment resulted in a decrease in survival of shoot tips (genotype S3-104) with longer times of incubation in PVS2 (Figure 5.3). A second experiment using genotype S3-109 gave similar results (Figure 5.4). Shorter times of exposure to PVS2 (less than 30 min) seemed to have no dramatic effect on survival (Figure 5.5).

Figure 5.3. Survival of shoot tips of genotype S3-104 after 20 - 100 min of incubation in PVS2 solution.

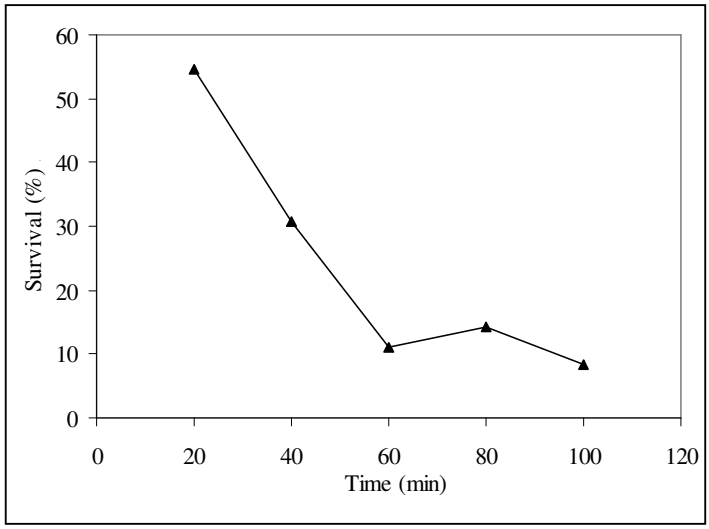


Figure 5.4. Survival of shoot tips of genotype S3-109 after 20 - 100 min of incubation in PVS2 solution.

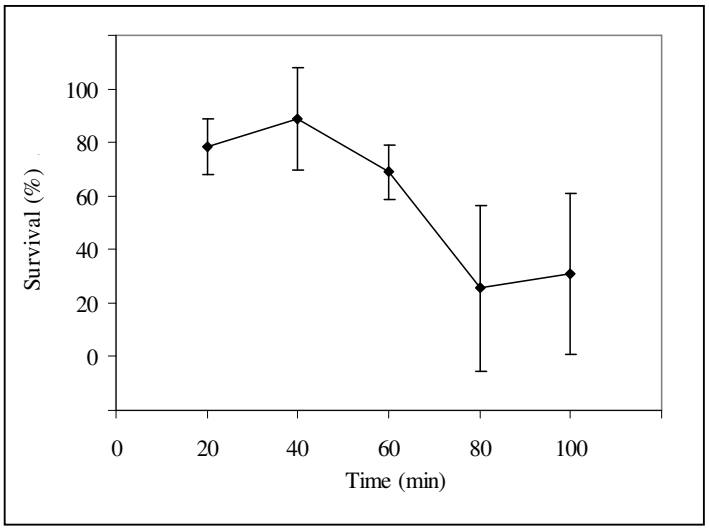
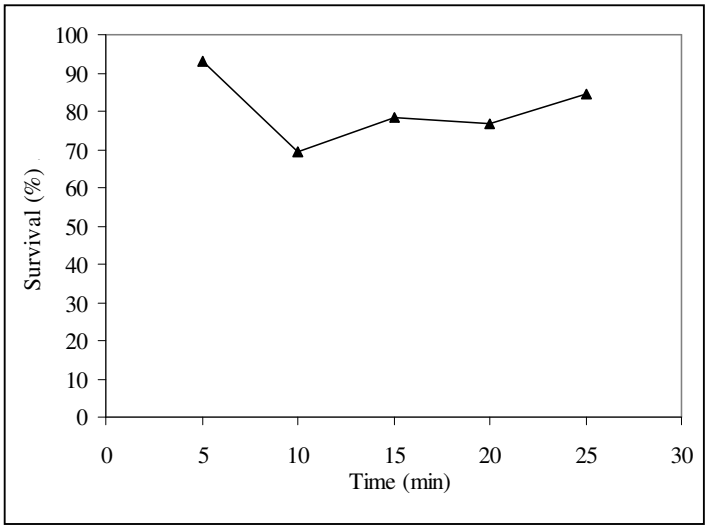


Figure 5.5. Survival of shoot tips of genotype S3-104 after 5 - 25 min of incubation in PVS2 solution.



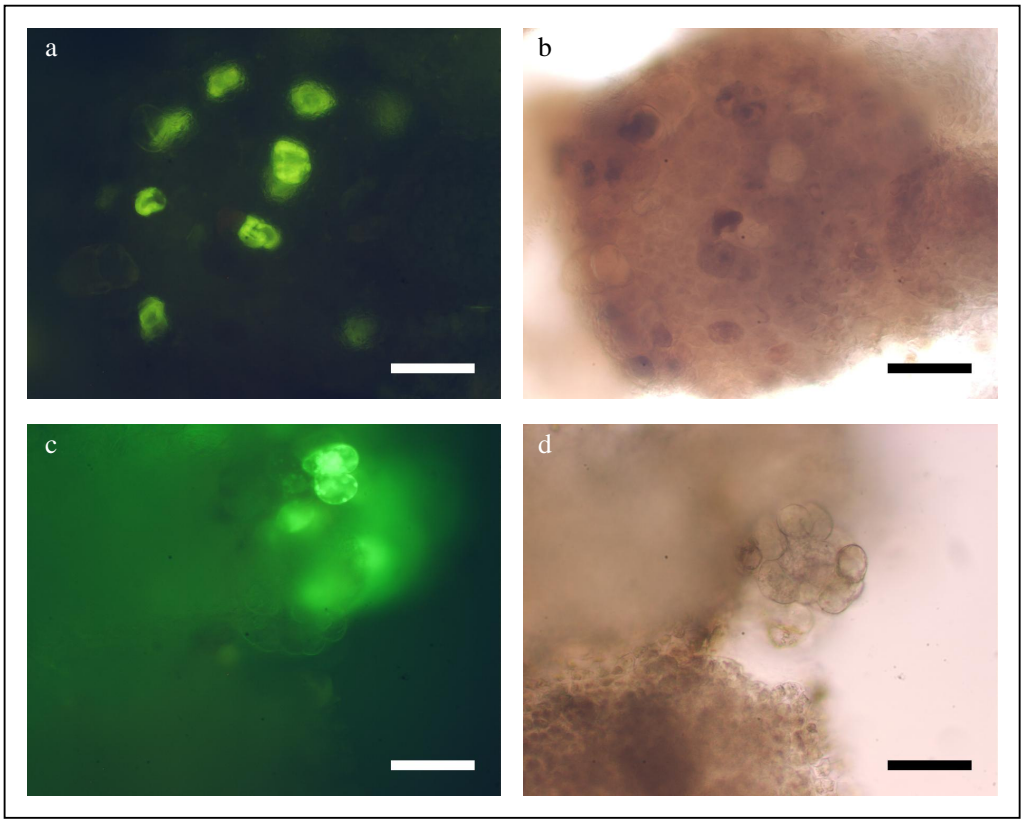
The experiment using a water bath at 3 temperatures for the thawing step and the experiment evaluating preculture treatments, resulted shoot tips not surviving.

The slow-cooling technique using shoot tips and the experiment using a direct plunge in LN with different vitrification solutions (PVS2 or VSL) gave no living shoot tips. Similarly, the experiments using the optimized time of exposure to PVS2, changes of solutions to wash thoroughly the explants from the previous treatments, and a final incubation in a high sucrose containing medium in the dark, gave no living shoot tips after storage in LN.

5.3.1.2 Cryopreservation of embryogenic callus

In the first experiment using vitrification and one-step freezing, surviving cells of genotype S3-172 were observed after 47 d of storage in LN and incubation on semi-solid medium (Figure 5.6a). The use of a slow-cooling procedure gave similar results (Figure 5.6c, d) and the embryogenic callus proliferated after 3 months. The experiment to assess the encapsulation-vitrification technique gave no surviving cells, although the control (no LN treatment) recovered and proliferated.

Figure 5.6. Surviving cells of embryogenic callus 47 d after cryopreservation.



a) Living cells after storage in LN using one-step freezing, stained with FDA and observed under UV light.

b) Same explant shown in a) but viewed with bright field illumination.

c) Living cells after storage in LN using the slow-cooling technique, stained with FDA and observed under UV light.

d) Same explant shown in c) observed with bright field illumination.

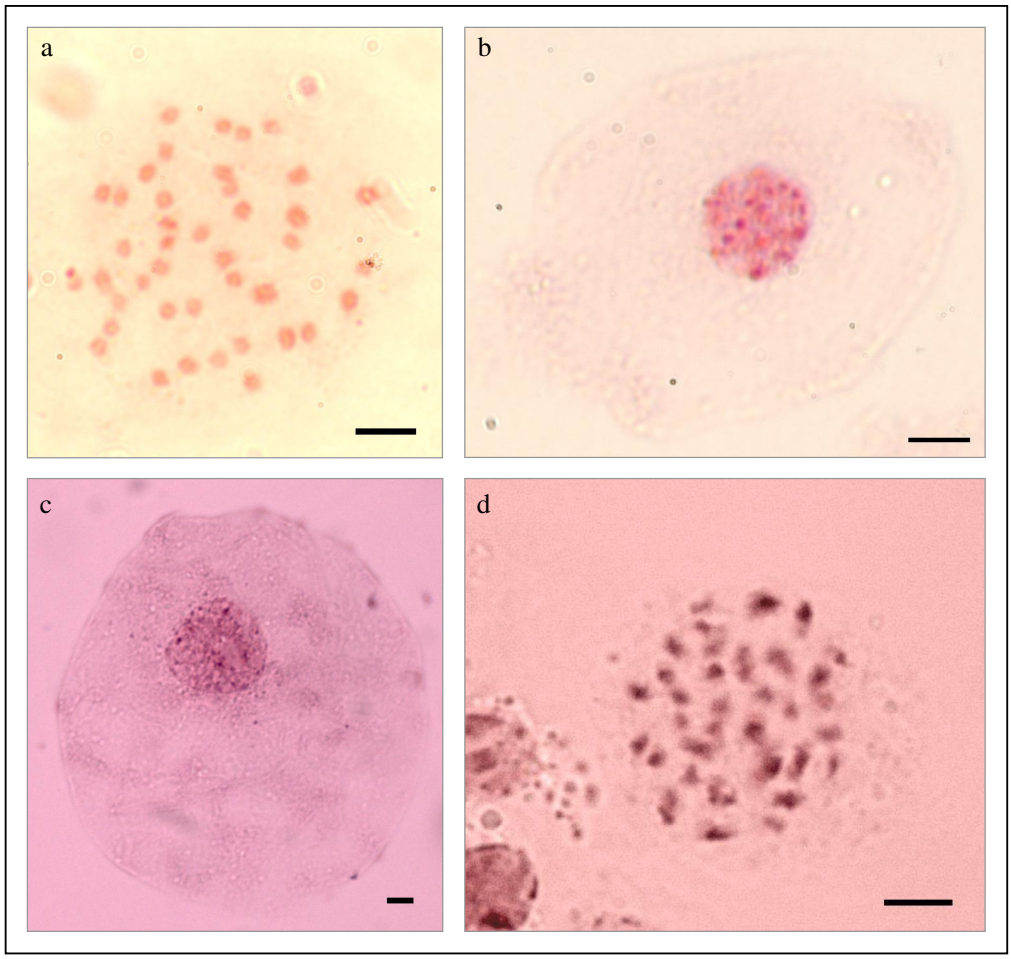
Scale bars = 25 µm (all images).

5.3.2 Cytological observations

Preparations from embryogenic cultures in liquid medium (genotype S3-112) provided the optimum material for chromosome observation, with abundant, actively dividing cells (Figure 5.7a). Embryogenic callus of genotype S3-172 showed a few cells with visible chromosomes, but the latter could not be counted (Figure 5.7b). Cells from callus of genotype B3-2853 showed no visible chromosomes (Figure 5.7c). A few cells (6) of embryogenic callus recovered after cryopreservation had visible chromosomes, but the latter could not be counted with confidence (Figure 5.7d). Preparations of root tips from *in vitro* material revealed a few cells with identifiable chromosomes but the latter were difficult or impossible to count (Figure 5.8). Chromosomes could not be observed in root tips of acclimatized plants.

The number of chromosomes counted for the embryogenic cultures of genotype S3-112 was variable and, in the majority of the cases, was near to 42, with a few cases with greater numbers (Figure 5.9).

Figure 5.7. Visualisation of chromosomes stained with aceto-orcein.



a) Chromosomes from a cell of genotype S3-112 cultured in liquid medium.

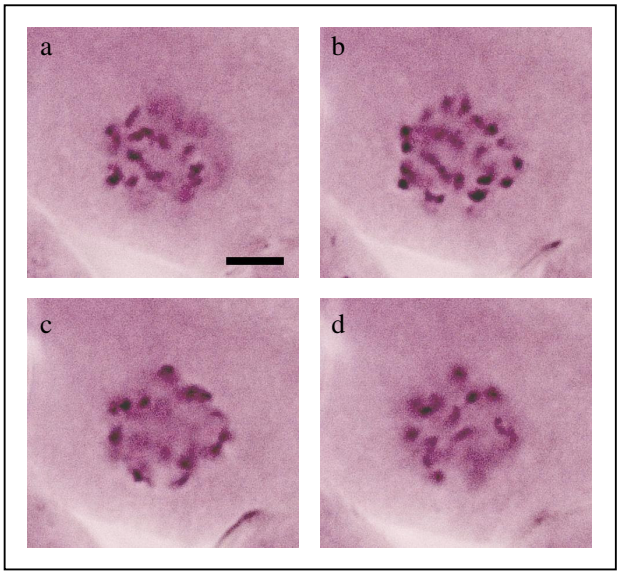
b) Cell of genotype S3-172 cultured on semi-solid medium.

c) Cell of callus (genotype B3-2853) cultured in liquid medium.

d) Chromosomes of genotype S3-172 in a cell recovered after cryopreservation.

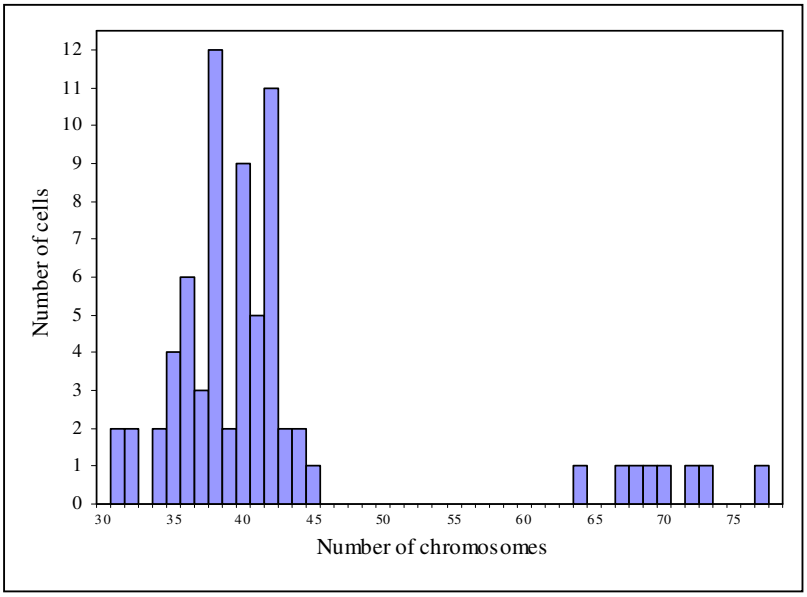
Scale bars = 4 µm (all images).

Figure 5.8. Sequence of photographs of an *in vitro* root tip cell.



From a) to d), the sequence shows chromosomes at different focus depths in a single cell. Scale bar = 5 µm.

Figure 5.9. Histogram of chromosome numbers in cells from embryogenic cultures in liquid medium.



5.4 Discussion

5.4.1 Cryopreservation

5.4.1.1 Cryopreservation of shoot tips

In the first experiment, the survival of shoot tips after incubation in loading solution indicated that preculture on sucrose-enriched (0.3 M) WP medium and the loading solution were harmless to the plant material. Fifty per cent survival of the shoot tips treated with PVS2 suggested that this solution had some toxic effect on the shoots. The sucrose concentration in the unloading solution showed no influence on the survival of shoots of *G. keule*.

Experiments to test survival after different times of exposure to PVS2 confirmed that this solution was toxic to explants of *G. keule*. This toxicity is well known for several species (Sakai and Engelmann 2007; Benson 2008). Variation in the survival after PVS2 exposure may be attributed to genotype, quality of the plant material and number of replicates. It was considered that the time of exposure to PVS2 longer than 60 min resulted in loss of shoot tip survival. It is known that incubation in vitrification solution allows the cryoprotectants to enter the cells and exert their beneficial effect during freezing and thawing, but as the toxicity of the solution increases with the incubation time, it is fundamental to optimize the time of incubation (Lambardi *et al*. 2008).

In the experiment testing 3 temperatures of the water bath, shoot survival was not observed, presumably attributed to the freezing step applied. However, controls from other experiments without storage in LN survived the 2 min incubation in the water bath at 35°C, indicating that this temperature is not harmful for shoot tips of *G. keule*.

Preculture of shoot tips on sucrose-enriched (0.3 M) WP medium for 18 d in the second experiment to evaluate PVS2 exposure, did not affect the survival of shoot tips since the control, taken after the preculture stage, showed 100 % survival (10 shoot tips). This provided a base for the experiment to evaluate preculture treatments. However, survival of shoot tips was not obtained from those experiments, indicating that the storage in LN was lethal. Benson *et al*. (2006) described preculture on medium with high content of sucrose, which may be combined with low temperatures, improving recovery from cryostorage. Using those treatments in *G. keule*, no surviving shoots after cryopreservation were observed.

The slow-cooling technique was lethal for shoot tips of *G. keule*, as is the case for the shoot tips of several tropical species (Gonzalez-Arnao *et al*. 2008).

All controls survived in experiments using the optimized time of exposure to PVS2 after 12 d of being treated, suggesting that the freezing step is responsible for death of explants. It is known that variation in survival after cryopreservation is influenced by plant genotype, physiological status (*in vitro* age of cultures, shoot location, frequency of subculture), protocol and culture conditions, such as light and PGRs (Harding *et al*. 2009).

However, for all procedures, genotype and culture conditions assayed in *G. keule*, storage in LN resulted in the failure of shoot tips to recover. This was attributed specifically to the storage in LN step, because controls that were not plunged in LN survived following different protocols. Interestingly, some cells were alive after cryostorage (Figure 5.1), but tissue proliferation was not observed. In olive, a similar situation has been reported, where successful recovery of shoot tips after cryopreservation was not present, although shoot tips survived and produced callus after freezing in LN (Lynch *et al*. 2007). This suggests that for some species the techniques now available are not suitable for cryostorage. Apparently, this is the case of *G. keule*, since shoot tips were not able survive LN storage using either slow-cooling or one-step freezing.

Cultures of living shoots observed after more than 1 year without subculture for several genotypes indicated that the medium-term storage using a slow growth strategy may constitute a valuable alternative for the conservation of shoot tips of *G. keule*. This method is currently used for the conservation of endangered species (Paunescu 2009).

5.4.1.2 Cryopreservation of embryogenic callus

Embryogenic callus showed some living cells after cryostorage (Figure 5.6a). Although many cells were dead (Figure 5.6b), the presence of living cells gave the first signs of successful cryostorage in *G. keule*. Moreover, proliferating embryogenic callus was recovered from cryostored material. This will allow the long-term storage of germplasm of *G. keule*, providing an important means of *ex situ* conservation for this endangered species.

Cryopreservation of somatic embryos is well documented in *Citrus* (Gonzalez-Arnao *et al*. 2008) as well as in temperate trees of the genus *Picea*, *Pinus*, *Aesculus* and *Quercus*, and in tropical perennial herbs, such as *Musa* and *Saccharum* (Lambardi *et al*. 2008). In *Vitis spp*., the cryopreservation of embryogenic cultures in liquid medium is considered a valuable tool for the storage of material suitable for genetic transformation (González-Benito *et al*. 2009).

In the experiments using encapsulation-vitrification of alginate beads, living cells were not observed after cryostorage. However, callus recovered and proliferated in the control, showing that the freezing step was lethal and the tissues survived the encapsulation treatment. It is possible that the time of incubation in PVS2 was not enough for the vitrification solution to diffuse through the alginate bead. Also, because the beads have a larger volume than the plant tissues, the large final volume (beads and PVS2) may have delayed freezing and the thawing process resulting in a more pronounced damage to the cells.

Cryopreservation of seeds of *G. keule* has not been attempted, since their scarcity and difficult germination make their use less feasible than embryogenic tissues. This alternative has also been suggested for endangered palms (Sarasan *et al*. 2005).

5.4.2 Cytological observations

The best material to observe chromosomes were small embryogenic aggregates of genotype S3-112. Chromosome count of these cells showed variable numbers around 42 (Figure 5.9), which is the reported diploid number for *G. keule* (Goldblatt 1976; Baeza *et al*. 2001). The small size of the chromosomes and their disposition at different depths in the material, which hampered the observation of all chromosomes at a single depth of focus, were likely to cause miscounting. The lack of appropriate amounts of material was also a constraint. The majority of cells seemed to be diploid, although some of them showed more chromosomes, probably corresponding to abnormal polyploid cells. The presence of polyploid cells in cultured tissues is well known (Phillips *et al*. 1994). Cytological abnormalities have been reported to cease after the regeneration of plants from such materials (Vargas *et al*. 2008).

Because of the scarcity of material and the low frequency of cells in metaphase, chromosome counts were even less confidently performed for root tips and cryopreserved material. The basic number of chromosomes seemed to agree with previous reports in *G. keule* (Goldblatt

1976; Baeza *et al*. 2001), but could not be confirmed with confidence. Chromosome morphology could not be observed because of their small size.

The difficulty of cytological observations in the present study suggested that chromosome observation is not a reliable method to assess genetic stability in cultured material or regenerated plants of *G. keule*. This agrees with previous work (Rani and Raina 2000; Harding 2004) indicating that cytological analysis presents severe limitations when the number of chromosomes is high or the chromosome size is too small for detecting structural changes, both conditions present in *G. keule*.

5.5 Conclusions

Embryogenic callus showed surviving cells after cryopreservation using either controlled freezing or a procedure involving direct plunge in LN. Proliferation of the recovered embryogenic callus was observed after 3 months of cryopreservation. The cryostorage of embryogenic tissues will complement long-term conservation efforts for *G. keule*.

After the storage of shoot tips of *G. keule* in LN, regardless of the procedure applied, isolated cells survived but explant growth did not resume. Preculture treatments, the temperature used in thawing and sucrose concentration in the unloading solution, apparently did not affect the survival of shoot tips. Shoot tips of *G. keule* showed optimum survival after 60 - 80 min of exposure to PVS2, but longer incubation times resulted in increased toxicity.

Chromosomes were observed in cells from embryogenic cultures in liquid medium, which were the best material for this purpose. Chromosome numbers suggested diploidy in the majority of cases, with some polyploid cells. Observation of chromosomes from other tissues, including root tips, was difficult or impossible, preventing determination of the chromosome number.

Chapter 6

Molecular characterization of *Gomortega keule* using microsatellites

6.1 Introduction

6.1.1 The use of microsatellites in plants

Microsatellites are tandem repeats of short nucleotide sequence motifs (1 to 6 base pairs) which are randomly distributed and abundant throughout eukaryotic genomes. They are also termed simple sequence repeats (SSRs), short tandem repeats (STRs) and sequence-tagged microsatellites sites (STMS). The number of repeat units is highly variable among individuals, while the sequence flanking each microsatellite is well conserved (Holton 2001; Mohler and Schwarz 2004). The design of primers for those flanking regions allows the use of PCR to amplify the fragments, which are later size-analysed. The main cause of the natural variation in the number of repeat units is considered to be the slippage of polymerase during DNA replication, although there is still debate on the underlying mutation model, *i.e.* the infinite allele model or the stepwise mutation model (Kumar *et al*. 2009). Importantly, microsatellites show high polymorphism (multiple alleles) and are inherited in a co-dominant manner (Holton 2001). Additional advantages of microsatellites are their high reproducibility and the fact that the analysis does not require high quality DNA (Kumar *et al*. 2009). Analysis of microsatellites is usually undertaken using automated DNA sequencing instruments (Mohler and Schwarz 2004). The use of fluorescent labelled microsatellite primers and laser detection in genotyping procedures with an automated sequencer, has improved the throughput and automatisation, although the assay can be costly due to the high price of the fluorescent label (Agarwal *et al*. 2008).

Unfortunately, microsatellites are generally not transferable to related plant genera (Mohler and Schwarz 2004), or amplify poorly for related species (Kumar *et al*. 2009). The isolation of useful loci is usually a time-consuming and expensive process involving the creation of a genomic library, its screening by hybridization, DNA sequencing of positive clones, primer design, PCR analysis and identification of polymorphisms (Holton 2001). Also, there may be the occurrence of null alleles due to failure of amplification by PCR, which is usually attributed to mutations in the primer annealing sites. The presence of null alleles can lead to errors in genotype scoring, to a biased estimation of allelic and genotypic frequencies and to an underestimate of heterozygosity. Underestimation of genetic divergence can also occur when different forward and backward mutations produce alleles of the same size, but different origins (homoplasy). A common problem during the interpretation of fragment size is the appearance of stutter peaks or bands caused by slippage during PCR amplification. Heterozygotes with two similar allele sizes can be difficult to distinguish from homozygotes, if there a large numbers of stutter bands are present (Kumar *et al*. 2009).

The high level of polymorphism of microsatellites gives rich information to be used in population studies (Kumar *et al*. 2009). The genetic diversity and mating systems in tree species, particularly the genetic differentiation among populations, are important for conservation of genetic resources. Microsatellites give information of gene flow within and among populations. They allow the study of selfing rate, biparental inbreeding, the differential paternal contribution of each individual tree to future populations and the fine-scale genetic structure within populations. Efficient management systems can be determined for conservation or timber exploitation by understanding pollen flow (Tsumura *et al*. 2004).

In addition, the properties of microsatellites make them ideal markers for plant breeding (Mohler and Schwarz 2004) and gene mapping studies (Kumar *et al*. 2009). They are also useful to assess the genetic variation present in germplasm collections, to identify genotypes or clones (Kumar *et al*. 2009) and to check the genetic stability of cultured materials. Different molecular markers have been used to analyse the genetic fidelity of *in vitro* cultured material in herbaceous plants like *Chlorophytum arundinaceum* (Lattoo *et al*. 2006), *Hypericum perforatum* (Urbanová *et al*. 2006) and *Swertia spp*. (Chaudhuri *et al*. 2007; Joshi and Dhawan 2007), and in trees such as *Cedrus spp*. (Renau-Morata *et al*. 2005), *Betula pendula* (Ryynänen and Aronen 2005), *Platanus acerifolia* (Huang *et al*. 2009), *Robinia ambigua* (Guo *et al*. 2006). Specifically, microsatellites have been employed to study somaclonal variation in trees such as *Olea spp*. (Lopes *et al*. 2009), *Picea abies* (Harvengt *et al*. 2001), *Pinus pinaster* (Marum *et al*. 2009) and *Quercus suber* (Santos *et al*. 2007).

6.1.2 Opportunities to apply microsatellites to *Gomortega keule*

Given the economic potential of *G. keule*, and having developed suitable methods for *in vitro* propagation of selected genotypes, it is important to assess any possible genetic variation during micropropagation. Microsatellites may provide a valuable first test.

As there is so little information about the reproductive system of *G. keule*, a second question that can be explored using microsatellites relates to the genetic composition of an offspring and its mother tree. This might provide knowledge of the pollination régime (self or cross-pollination), as in studies in the trees, *Caryocar brasiliense* (Collevatti *et al*. 2001), *Cryptomeria japonica* (Moriguchi *et al*. 2005) and *Vitex lucens* (Barrel *et al*. 1997). In the previous dissection of seeds made in this research for *in vitro* culture, it was observed that there are 2 storage tissues. The external and most abundant was thought to be perisperm, which would have the same genotype as that of the seed's mother tree. A second and internal tissue located between the embryo cotyledons was thought to be endosperm. Both storage tissues are visible in an anatomical photograph published by Heo *et al*. (2004), but the authors did not refer to these structures.

A third question that can be addressed with the use of microsatellites in *G. keule*, relates to the genetic characteristics of natural populations. This is important for conservation purposes, complementing previous studies (García-Gonzáles *et al*. 2008), and from the point of view of plant genetic resources involving future work using germplasm with selected characteristics.

The objectives of the chapter were:
1) To evaluate the genetic fidelity of cultured materials using microsatellites.
2) To explore the genetic composition of seed tissues.
3) To assess the genetic variation in natural populations of *G. keule*.

6.2 Materials and methods

6.2.1 Plant materials

Plant material was collected from the VIIth Region of Maule and the VIIIth Region of Biobío, Chile (Table 2.1). At Reserva Nacional Los Queules, 10 fresh fruits were collected from one recorded tree with the aim of studying its offspring. The material was sent to The University of Nottingham with the required phytosanitary certificates and arrived in 7 days (received on 5th June 2007). *In vitro* cultured material that originated from seeds collected on the forest floor at Ralbún in May and June of 2006 was used for evaluating genetic fidelity on 2 embryogenic lines (S3-112 and S3-172).

A field trip to the southern populations of *G. keule* in Arauco was carried out in December 2007. Samples from all the trees that could be found in the two populations visited (Figure 6.1), at a distance of 7.5 km from each other, were collected for the population study. Population Forestal Tierra Chilena comprises mainly very old trees, while Bosques Arauco had 6 trees growing from old stumps and the rest of the individuals were young trees or shoots growing under a *Pinus radiata* plantation, from stumps of trees cut before or at the plantation establishment, around 15 years ago. Cambium was sampled when young leaves could not be found for a tree. Tissues were kept in 32 ml plastic Universal tubes (Barloworld Scientific, Aberbargoed, UK) with orange drying pearls (Sigma) which were changed and heat-reactivated until the sample was dry, indicated when the colour of the beads did not change. Once the material arrived in the laboratory, it was stored at -20°C to prevent degradation.

Figure 6.1. Photographs of trees of *Gomortega keule* in habitat.

a) A tree in the population Forestal Tierra Chilena, the only population with old trees.

b) Young tree growing in a *Pinus radiata* plantation in the population Bosques Arauco.

6.2.2 DNA extraction

Extraction of DNA was evaluated using different methods; the preparation of solutions is detailed in Appendix 9.20. Immediately before DNA extraction and following modifications of Lander *et al*. (2007), 5 mg diethyldithiocarbamic sodium salt (Sigma), 10 mg PVP 40,000 (Sigma Co., St. Louis, USA) and 5 µl β-mercaptoethanol (Sigma) were added per 1 ml of CTAB solution pre-heated to 65ºC in a dry bath and dissolved by shaking.

Extraction of DNA from *G. keule* material cultured *in vitro* was attempted by modifying the procedure of Lander *et al*. (2007) by the use of GenElute Plant Genomic DNA Miniprep Kit (Sigma). The tissue was frozen in liquid nitrogen and ground using a sterile plastic micro-pestle in a 1.5 ml centrifuge tube. CTAB solution (700 µl) was added to the sample and mixed vigorously by vortexing. After 20 min incubation at 65ºC, 100 µl Lysis Solution Part B was

added. After a second incubation for 20 min at 65ºC, 500 µl of chloroform:isoamylalcohol was added, the tube vortexed and centrifuged for 5 min at 13,000 rpm. The supernatant was carefully pipetted and placed into a new tube. A second chloroform extraction was applied and 130 µl Precipitation Solution was added. From this point, the protocol followed the Sigma GenElute Plant Genomic DNA protocol.

Modifications of the previous protocol were applied by grinding the plant tissues with a pestle and mortar, using 500 µl of CTAB solution, 250 µl of Lysis Solution Part A, 35 µl of Lysis Solution Part B and then following the Sigma GenElute Plant Genomic DNA protocol. The latter protocol was again modified using 400 µl of CTAB solution, 200 µl of chloroform and centrifuging 2 min at 13,000 rpm after each chloroform extraction. DNA extractions were also attempted following the Sigma GenElute Plant Genomic DNA protocol and the QIAGEN DNeasy Plant Mini Kit protocol without modifications. Additionally, the procedure developed by Lander *et al*. (2007) was evaluated, using 500 µl CTAB.

Finally, the DNA extraction procedure was performed according to Rogers and Bendich (1985). Fresh tissue from cultures (100 mg) was ground in liquid nitrogen using a pestle and mortar. Mercaptoethanol (0.5 µl) was added to 100 µl 1x CTAB pre-heated to 65ºC, the mixture was added, mixed with the sample by pipetting, and incubated for 3 min at 65ºC. RNase was added (4 µl, QIAGEN DNeasy Plant Mini Kit) and incubated for 20 min at 65ºC. Chloroform (100 µl) was added and mixed vigorously, then centrifuged for 2 min at 13,000 rpm. The supernatant was transferred to a new tube and 10 µl 10x CTAB added and mixed vigorously. A second chloroform extraction was performed. CTAB precipitation buffer (100 µl) was added and mixed. Then 200 µl high salt TE buffer were added and mixed, followed by pure ethanol being added (400 µl, Fisher), mixed and centrifuged for 5 min at 13,000 rpm. The supernatant was discarded and 800 µl of 80% (v/v) ethanol was added and mixed vigorously. After centrifugation for 5 min at 13,000 rpm, the supernatant was discarded and the sample left to dry overnight in a flow cabinet.

The method of Lander *et al*. (2007) was used as the regular procedure to extract DNA because it consistently yielded good quality DNA or, especially from dried samples, DNA which was later susceptible to amplification by PCR, despite some degree of degradation. DNA extraction was repeated even when no DNA was found, and as many times as the sampled tissue was available.

To extract DNA from seed tissues, endocarps were opened using a bench vice and seeds excised to obtain the embryo and the two storage tissues present in the seed. One of the 10

opened endocarps bore 2 seeds, so 11 seeds were excised. Zygotic embryos and soft *in vitro* cultured material (embryogenic cultures in liquid medium) were ground using a micropestle and centrifuge tube.

6.2.3 Development of new microsatellite primers

Primers for microsatellites CS2 and CS8 were developed by Dr. S. Mayes in the Plant and Crop Sciences Division, School of Biosciences, University of Nottingham, following the method reported by Edwards *et al*. (1996) and modified by Haddrill *et al.* (2002). The technique involves the digestion and PCR amplification of genomic DNA, hybridisation to filters with SSRs, elution and amplification. Rather than cloning, a mixture of enriched libraries were pyrosequenced (454 Life Sciences, Branford, USA) using a $1/16^{th}$ run (non-Titanium reagents).

The construction of enriched microsatellite libraries began with the preparation of filters, attaching a mixture of SSR oligonucleotides to nylon membranes by UV light. Genomic DNA was digested with restriction enzymes and adapters were ligated. PCR reaction using primers homologous to the adapters was performed for pre-enrichment. Products from the pre-enrichment reaction were allowed to hybridise to the filters for the enrichment of microsatellite sequences. The hybridised DNA from the membrane was eluted and amplified by PCR. The PCR products were separated by gel electrophoresis and sizes from 100 to 500 bp were excised and purified. The purified PCR products from a number of different species post-enrichment amplifications were mixed and sent for 454 sequencing. Adaptors with library-specific base differences were used to sort the returned sequence into species-specific libraries. Primers were designed, where possible, for the sequences flanking the microsatellite, were tested for PCR amplification and the optimum annealing temperature was screened (Mayes, unpublished data).

6.2.4 Gel electrophoresis

The preparation of solutions is detailed in Appendix 9.21. The quality of the extracted DNA was evaluated using a NanoDrop® Nd-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). To visualise DNA and to have an approximation of its concentration,

a 1% (w/v) agarose (SeaKem® LE Agarose; Lonza, Rockland, USA) gel was prepared with 1x TAE buffer and ethidium bromide (Fisher) was added to a final concentration of 0.5 µg/ml. Template DNA (2 µl) was mixed with 0.5 µl loading buffer (Blue/Orange 6x Loading Dye; Promega, Madison, USA) and loaded in the gel. Lambda DNA (10 and 50 ng, New England Biolabs Inc., Ipswich, USA) mixed with loading buffer (1:9) was also loaded on the gel, which was run for 2 h at 120 V.

To visualise amplification of DNA fragments using non labeled primers, a 2% (w/v) agarose gel was prepared with 1x TAE buffer, and ethidium bromide (Fisher) was added to a final concentration of 0.5 µg/ml. PCR products (5 µl) were loaded into each of the gel wells. Also, 3 µl of ladder (2-Log DNA Ladder, New England Biolabs) mixed with 2 µl loading buffer were loaded onto the gel, which was run for 40 min at 90 V.

PCR products with fluorescently labeled primers were visualised in a gel prepared with 2% (w/v) agarose (Ultra Pure™ Agarose; Invitrogen, Paisley, UK) and 0.5x TBE buffer. Ethidium bromide (solution 10 mg/ml, Promega) was added to a final concentration of 0.1 µg/ml. PCR products (3 µl) were mixed by pipetting with 3 µl loading buffer (prepared as detailed in Appendix 9.22) and the mixture loaded into the gel well. Also, 2-4 µl of ladder (ladder:loading buffer:water, 1:3:16) was loaded on the gel, which was run for 42 min at 130 V.

6.2.5 PCR amplification of microsatellites

Primers for 8 microsatellites, designed by Lander *et al*. (2007), were obtained from Eurofins MWG Operon (Ebersberg, Germany) and were dissolved in sterile reverse-osmosis water to obtain 100 mM stock solutions. Working solutions (10 mM) were prepared from the latter. A 0.1 µg/µl working solution of BSA (Promega) was prepared. A 10 µl PCR contained 2 µl template DNA, 0.25 µg BSA, 2 µl PCR buffer (5x Green GoTaq® Flexi Buffer; Promega), 0.6 µl (Gk-6), 0.8 µl (Gk-30 and Gk-39) or 1 µl (Gk-44) of 25 mM $MgCl_2$ (Promega), 0.2 µl dNTP (10 mM, Sigma), 0.2 µl (for Gk-6) or 0.4 µl (for Gk-30, Gk-39 and Gk-44) working solution of forward and reverse primers, and 0.2 µl *Taq* polymerase (GoTaq® Flexi DNA Polymerase; Promega). The reaction conditions programmed in a Gene Amp® PCR System 9700 (Applied Biosystems) were 94°C 3 min, 30 cycles of 94°C 1 min, 61°C 1 min, 72°C 1 min, with a final extension of 72°C 20 min.

Forward primers were labeled with a blue, green or black fluorescent dye (WellRED Primers; Sigma) and suspended in 1x TE buffer (detail of preparation in Appendix 9.22) to obtain a 100 µM solution.

For each labeled primer, the optimum annealing temperature for PCR was assessed in a Px2 Thermal Cycler (Thermo Fisher Scientific Inc.) programmed with 12 temperatures ranging from 50 to 65°C. Template DNA (10 ng/µl) was prepared by pooling equivalent quantities from 6 samples of different types of tissues (embryogenic cultures, endosperm and dried leaves). PCR (20 µl reaction volume) was performed using 2 µl template DNA, 2 µl buffer (Standard Taq Reaction Buffer, New England Biolabs), 0.2 µl dNTP, 0.05 µl BSA (10 µg/µl, New England Biolabs), 0.02 µl of each forward (fluorescent labeled) and reverse primers, 15.53 µl pure autoclaved H_2O and 0.2 µl *Taq* polymerase. Master mixes for multiple reactions were made wherever possible. The reaction conditions were 94°C 3 min, 30 cycles of 94°C 1 min, 1 min at the specific annealing temperature tested, 72°C 1 min, and a final extension of 72°C 20 min.

PCR with the labeled primers (20 µl reaction volume) was performed using 2 µl template DNA, 2.5 µl buffer, 0.2 µl dNTP, 0.05 µl BSA (10 µg/µl), 0.02 µl of each forward (labeled) and reverse primers, 15 µl pure autoclaved H_2O and 0.2 µl *Taq* polymerase. Master mixes for multiple reactions were made wherever possible. The reaction conditions were 94°C 3 min, 30 cycles of 94°C 1 min, 1 min at the specific annealing temperature of each primer, 72°C 1 min, and final extension at 72°C for 20min. The annealing temperatures were 60.9 (Gk-1), 55.4 (Gk-6), 65.0 (Gk-5, Gk-30, Gk-31 and Gk-35), 56.8 (Gk-39) and 52.7°C (Gk-44, CS2 and CS8).

Primers Gk5 and Gk44 showed very poor amplification for dried sample-derived DNA and, consequently, they were not included in the population study. However, amplification was satisfactory for samples of *in vitro* cultures and zygotic embryos.

6.2.6 Analysis of PCR products by capillary electrophoresis

A total of 181 samples were analysed by capillary electrophoresis, 43 from the northern populations (10 of each 2 embryogenic cultures, 1 mother tree, external storage tissue and the embryos of 11 seeds), 75 from Bosques Arauco and 63 from Forestal Tierra Chilena.

The PCR products were pooled according to their different fluorescent tags, with a maxium of 3 samples of different colours per well to be analysed. The samples were mixed in proportions following the estimated DNA quantity of the PCR products (from gel electrophoresis) and previous experience by the staff in the Division of Plant and Crop Sciences (Table 6.1).

Before loading the samples in the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, USA) for fragment size analysis, 2 µl of the pooled samples were mixed with 3 µl of Sample Loading Solution (Beckman Coulter) and 0.25 µl of 400 bp Size Standard (Beckman Coulter), and a drop of mineral oil (Beckman Coulter) was added to the well.

Table 6.1. Volume of PCR product (µl) used to mix samples for analysis by capillary electrophoresis.

Dye colour	PCR product quantity [1]		
	Medium or high	Low	Very low
Blue	2	3	4
Black	8	11	13
Green	4.5	6	7

[1] Estimated as intensity on agarose gel.

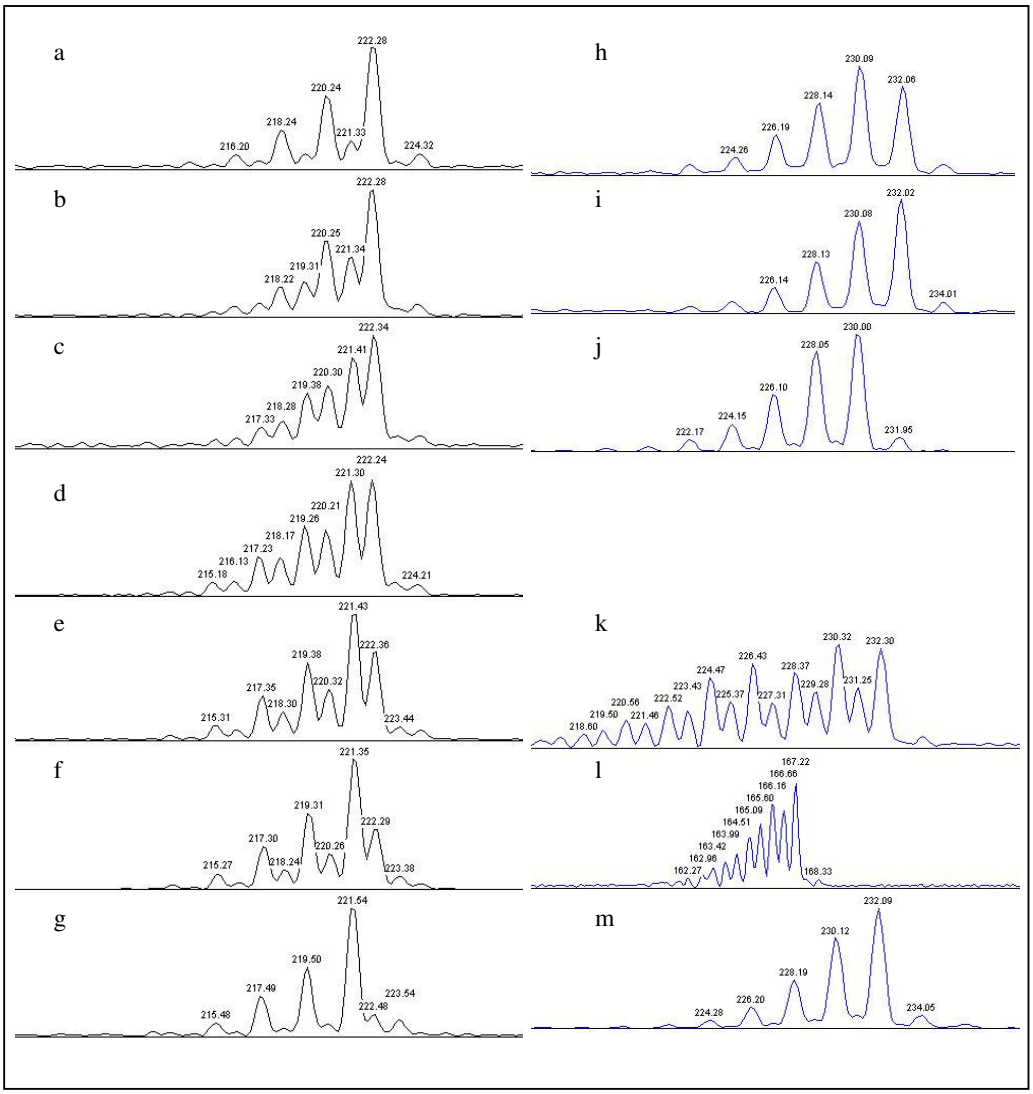
6.2.7 Calling of peaks from capillary electrophoresis

The peaks produced by capillary electrophoresis were examined thoroughly within each locus in order to be familiar with the shape of the peaks, their height relationships and size range. For each locus, and in some cases for individual alleles, criteria were set to consider peaks as valid alleles.

A first problem to call peaks was that, in many cases, groups of peaks showed a pattern with a major peak on the right being near an even number (Figure 6.2a), or a major peak on the left near the previous odd number (Figure 6.2g), or *vice versa*. There were also several samples with intermediate situations (Figure 6.2b to f), suggesting that the whole ensemble of peaks corresponded to only one allele, and the expression of the peak with the most intense signal near an even or odd number size was due to PCR artifacts. The fact that repeated PCRs showed changes in this pattern, but not in the size of the amplification products, supports this interpretation. Therefore, when an odd sized peak showed the more intense signal, the next upper even sized peak was called, or, with identical result, its binned size was forced to its

next upper even number (or *vice versa*). This situation was observed in many alleles for several of the microsatellites used and was always resolved under the same criteria. In the case of locus Gk-31 (Figure 6.2a to g), peaks around 222 bp were called even if its neighbour peak at 221 bp was higher, or, with identical result, its binned size was forced from 221 to 222.

Figure 6.2. Examples of peaks with difficult interpretation from capillary electrophoresis.



A gradient of a major peak at an even number size which decreases while its neighbour peak with an odd number size grows (a to g) suggests that the whole group of peaks corresponds to a single allele, as shown for alleles 221 and 222 of locus Gk-31. An heterozygotic sample (h) could be identified when the normal pattern of a single allele at locus Gk-1 showed a major peak on the right and stutter peaks sharply decreasing to the left (i, j), supported by the occurrence of samples with only one of the two alleles (i, j). A pattern that could not be interpreted (k) or out of size range (l) led to repeat PCR and capillary electrophoresis to obtain a proper pattern (m), as shown for sample 28 at locus Gk-1.

A second problem was the occurrence of heterozygotic samples with allele sizes 2 bp apart (Figure 6.2h). When the typical pattern had the peak with the most intense signal being the first on the right, as for microsatellite Gk-1 (Figure 6.2i, j), a heterozygote could be discriminated when the first peak on the right was not the one with the more intense signal. This situation was supported by the observation of homozygous samples for either one or the other allele (Figure 6.2i, j).

Additional problems with peak calling were the occurrence of patterns that were not possible to interpret (Figure 6.2k) and of patterns with sizes out of the expected range (Figure 6.2l). For both situations, PCR and capillary electrophoresis were repeated to obtain a pattern suitable for interpretation (Figure 6.2m).

6.2.8 Binning of alleles

Binning is the process of converting raw allele lengths (from calling) into allele classes of integer numbers (Amos *et al*. 2007). Called alleles were automatically binned using Flexibin (Amos *et al*. 2007) and graphs generated by this software (Figure 6.4) were studied to look for potentially miscalled alleles. When alleles at the edges of a category in the distribution graph were dubious, calling was checked to ensure that the output of the binning process was consistent.

Primers for microsatellite Gk-6 showed up to 4 alleles in a single sample, suggesting amplification for 2 loci. Assuming the occurrence of 2 loci, no presence of null alleles and alleles of different sizes in each locus, alleles were assigned to either one or the other of these 2 putative different loci. This was solved by looking first to samples showing 2 alleles, thus homozygous at both loci, and so these alleles were considered to be in different loci. Then, samples showing 3 alleles were observed and tables with alleles in the different loci were built. Finally, all the alleles could be assigned to the loci. For all 181 samples analysed, 17 different genotypes were found for these 2 loci. No sample showed a genotype bearing an allele from the other locus, which supports the assumptions. No sample showed none or only one allele, supporting the idea that null alleles did not occur for Gk-6.

6.2.9 Statistical analyses

Binary matrices were generated for the presence or absence of each allele at every locus, for all the samples. Samples from seeds (seed tissues study) and *in vitro* cultures (clone fidelity study) were analysed comparing their allele presence or absence for each of the 11 loci included. With the aim of building a group of samples from the North for the population study, one sample of each *in vitro* culture (2 genotypes originated from zygotic embryos from Ralbún), samples of the embryos and from the putative mother tree (from Reserva Nacional Los Queules) were included. Samples of the 2 populations from the North are referred to as population 'North' from here onwards. This group was included with the aim of estimating its relationship to the South populations but not of being characterized.

As reviewed by Laurentin (2009), when studying the genetic diversity of a group of accessions or samples, two important outcomes are possible, to quantify the genetic diversity, and to assess the genetic relationship among the elements. For the quantification of the genetic variability, polymorphic loci (those with the commonest stage with a frequency less than 0.95) and the unbiased estimate of heterozygosity (Nei 1978) are appropriate concepts for co-dominant markers (Laurentin 2009).

According to Laurentin (2009), in order to evaluate the genetic relationship within a group, association coefficients for binary data are calculated, the similarity index of Nei and Li (1979) being one of the most used. To visualise the relationships, cluster analysis and ordination methods are used as complimentary techniques since they have different theoretical basis. Cluster analysis classifies the individuals into groups in a hierarchical structure, being the unweighted pair-group method using arithmetic averages (UPGMA) the most frequently used algorithm. One of the most common methods of ordination used in assessment of the diversity of plant genetic resources is principal coordinate analysis (PCO) with Euclidean distances (Laurentin 2009). This method makes no assumptions about the distribution of the data or about their population genetics, while Euclidean distance does not class common absence of an allele as a shared characteristic (Kloda *et al*. 2008).

PCO and cluster analysis were performed using MVSP software (Multivariate Statistical Package version 3.13, Kovach Computing Services, Anglesey, UK). A graph was generated with the first two axes of eigenvalues and a dendrogram was produced by UPGMA using Nei and Li's index (Nei and Li 1979) as the measure of similarity. For both analyses, all 152

samples were included, 75 were from BA, 63 from FTC and 14 from North, which includes individuals with identical genetic profiles.

As mentioned, some of the samples showed no differences in the dendrogram (Figure 6.6) and their identical genotype was confirmed by the presence of the same alleles in the binary matrix. Those samples consisted of 15 groups with only one genotype within each group, 13 groups with 2 samples each, 1 group with 3 samples and one group with 5 samples. For samples with reliable information from the field and being less than 10 m apart from each other, they were considered as possibly being part of the same individual (stems or shoots from a single old and now disappeared tree). Therefore, 4 groups of 2 samples each, 1 of 3 samples and 1 group of 5 samples, were considered as single individuals and the redundant samples were not included for the statistical analysis of populations. Of a total of 142 samples included in the study, 72 were from BA, 56 from FTC and 14 from North. All of them were assessed for 9 loci, except one sample that had not been amplified for microsatellites CS2 and CS8; these data were included as missing in the input matrix for the analyses. MVSP does not intrinsically accept missing data, so these cells will have been replaced with a '0'. This is likely to underestimate relationships within in the dataset, should it have any effect.

The number of alleles per population and locus was obtained using the software Arlequin (version 3.11, Excoffier *et al*. 2005) and FSTAT (version 2.9.3.2, Goudet 1995), and the number of private alleles with GDA (Genetic Data Analysis version 1.0, Wier 1996). The observed (H_O) and expected heterozygosity (H_E) for each locus and population were computed with Arlequin using formulas from Nei (1987), while for the values corresponding to the combined populations, GDA (formulas from Nei 1978) and Powermarker (version 3.25, Liu and Muse 2005) were used with procedures from Wier (1996).

Diversity between groups using co-dominant markers can be assessed, according to Laurentin (2009), by methodologies based on allele frequencies, such as Wright's *F*-statistics and Nei's parameters. Therefore, the inbreeding coefficient (F_{IS}) for each locus and population was computed using FSTAT according to Wier and Cockerham (1984), and for all populations with Powermarker (from Wright 1965). The fixation index (Rho_{ST} or ρ_{ST}) was calculated for each population pairwise using Genepop (version 4.0.5.3, Rousset 2008) with a procedure described by Michalakis and Excoffier (1996). A second version of the fixation index (F_{ST}) described by Slatkin (1995), also under the stepwise mutation model, was calculated using Arlequin.

Exact tests for Hardy-Weinberg equilibrium (HWE) were performed with Arlequin and Genepop software using the Markov chain method (forecasted chain length 1,000,000; dememorization steps 100,000). Linkage disequilibrium was tested separatedly for each population using Arlequin.

The frequency of null alleles was calculated assuming simultaneous inbreeding for each locus and population with the software INEst (Chybicki and Burczyk 2009) using either the population inbreeding model or the individual inbreeding model (1,000,000 iterations).

6.3 Results

6.3.1 DNA extraction, gel electrophoresis and PCR

DNA could be extracted by grinding the tissues with pestle and mortar and using different protocols (Table 6.2, Figure 6.3a). The protocol after Lander *et al*. (2007) yielded DNA from fresh tissue, which was well visualised. Visualisation of DNA from dried tissues showed a rather low quantity and degraded DNA (Figure 6.3a, lanes 7 and 8) in variable degrees (Figure 6.3c). Only 4 samples had no visible DNA in the gel after the extraction process and the samples were not subjected to PCR. All of these samples belonged to population FTC, one corresponded to cambium and 3 to young shoots or leaves. DNA from zygotic embryos showed no degradation (Figure 6.3d) and that from *in vitro* material in active growth was the most abundant (Figure 6.3e).

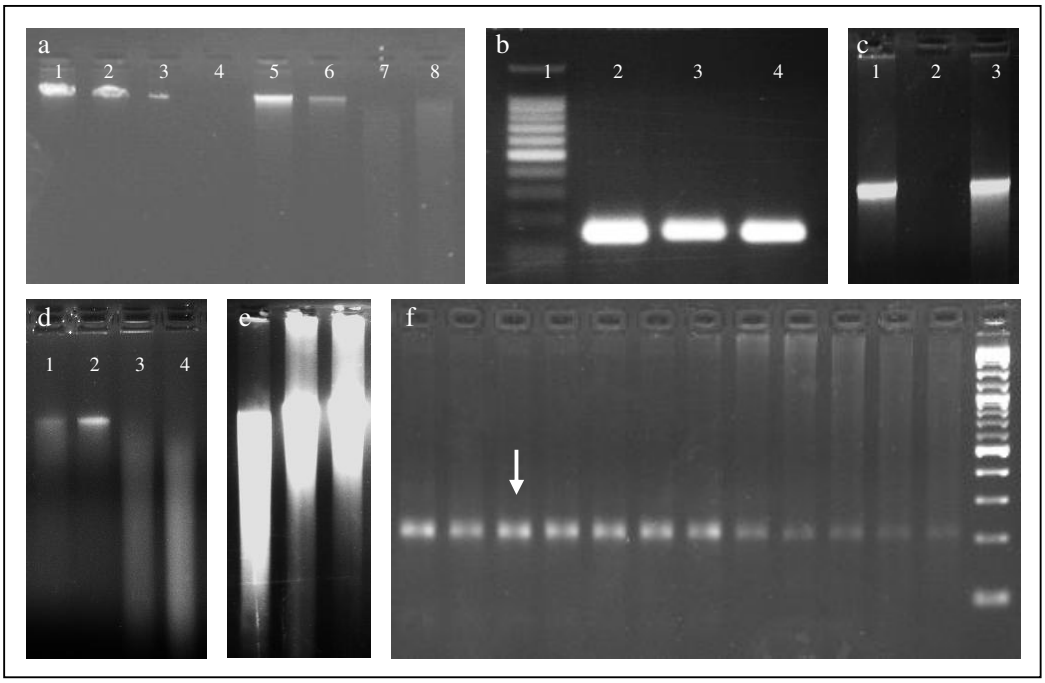
Preliminary, PCR amplified well using 4 pairs of primers (not labeled) and for fresh and dried tissue samples (Figure 6.3b). The amplification process was successful (as shown in lane 4 of Figure 6.3b) even when the template DNA used in the PCR did not have good quality, as seen by comparing lane 5 with 6 and 7 in the gel shown in Figure 6.3a. This enabled further work with dried samples collected from natural populations.

Table 6.2. Quality of DNA obtained using different extraction protocols.

Grinding procedure	DNA extraction protocol	Sample type	NanoDrop reading (ng/µl)	Visualisation in gel
Tube and micro pestle	Sigma kit	Fresh tissue	<5	Nothing
Tube and micro pestle	Lander *et al.* (2007) modified with Sigma kit	Fresh tissue	<0.5	Not performed
Pestle and mortar	Sigma kit	Fresh tissue	8-11	Strong
Pestle and mortar	Lander *et al.* (2007) modified with Sigma kit	Fresh tissue	<4	Not performed
Pestle and mortar	QIAGEN kit	Fresh tissue	17	Strong
Pestle and mortar	Lander *et al.* (2007)	Fresh tissue	2-20	Weak to strong
Pestle and mortar	Lander *et al.* (2007)	Dried tissue	5-8	Weak
Pestle and mortar	Rogers and Bendich (1985)	Fresh tissue	5-220	Nothing to very strong

Optimization of annealing temperature for labeled primers is illustrated in Figure 6.3f for microsatellite CS8. When amplifying with labeled primers rountinely, all samples showed PCR amplification for the majority of the labeled primers. Samples from dried tissues amplified erratically for microsatellites Gk-5 and Gk-44, even when a greater quantity of DNA template was used in the PCR reaction. Only one sample from dried tissue (number 265) did not amplify for microsatellites CS2 and CS8.

Figure 6.3. Gel electrophoresis for DNA visualisation.



a) DNA quality for different extraction protocols. Lanes 1, 2 and 3: lambda DNA (25, 10 and 5 ng respectively). 4: fresh tissue ground in tube extracted with Sigma kit. 5 and 6: fresh tissue ground with a pestle and mortar, extracted with Lander *et al.* (2007) protocol. 7 and 8: dried tissue ground with a pestle and mortar using the latter protocol.

b) PCR products using primer Gk-30. 1: ladder DNA. 2 and 3: amplification of DNA extracted from fresh tissue. 4: amplification of DNA from dried tissue.

c) DNA from seed tissues. 1: embryo. 2: inner storage tissue. 3: endosperm.

d) DNA from dried tissues. 1 and 2: cambium samples. 3 and 4: young leaf samples.

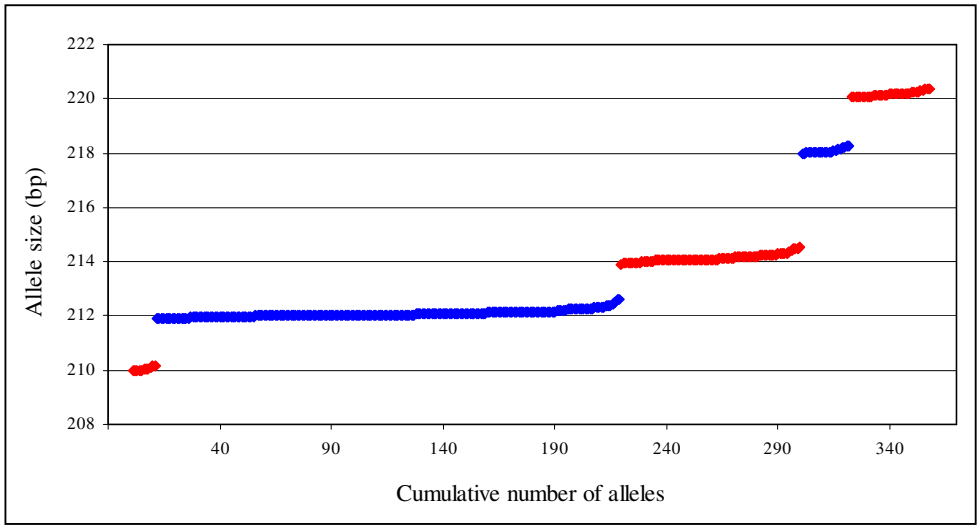
e) DNA extracted from embryogenic *in vitro* cultures.

f) PCR products for different annealing temperatures (primer CS8). The arrow indicates the temperature chosen as optimum (52.7°C).

6.3.2 Binning of alleles

Part of the binning process output is illustrated in Figure 6.4, where the cumulative length distribution is shown of microsatellite CS8 generated with Flexibin (Amos *et al*. 2007). For this microsatellite, the binning result was clear and all allele sizes were multiple of 2. Alleles found for each locus are presented in Table 6.3.

Figure 6.4. Cumulative length distribution of alleles for microsatellite CS8.



Five classes of alleles are distinguishable and coloured red and blue alternately.

Table 6.3. Alleles found for microsatellites of *Gomortega keule* (size in bp).

Gk-1	Gk-5	Gk-6 locus 1	Gk-6 locus 2	Gk-30	Gk-31	Gk-35	Gk-39	Gk-44	CS2	CS8
224	307	98	92	182	208	224	133	155	104	210
226	311	106	104	184	212	226	189	156	115	212
230	315	108	114	186	216	228	191	157	117	214
232	317	110	116	188	218	236	199	159	119	218
234	319		118	190	222	238	201			220
236			120		224	242				
240					226	252				
					228					

6.3.3 Clonal genetic fidelity

Within each of the 2 lines of embryogenic cultures, all the 10 samples showed the presence of the same alleles, *i.e.* a full matching or the same genetic identity was found for all the samples of each group of *in vitro* material.

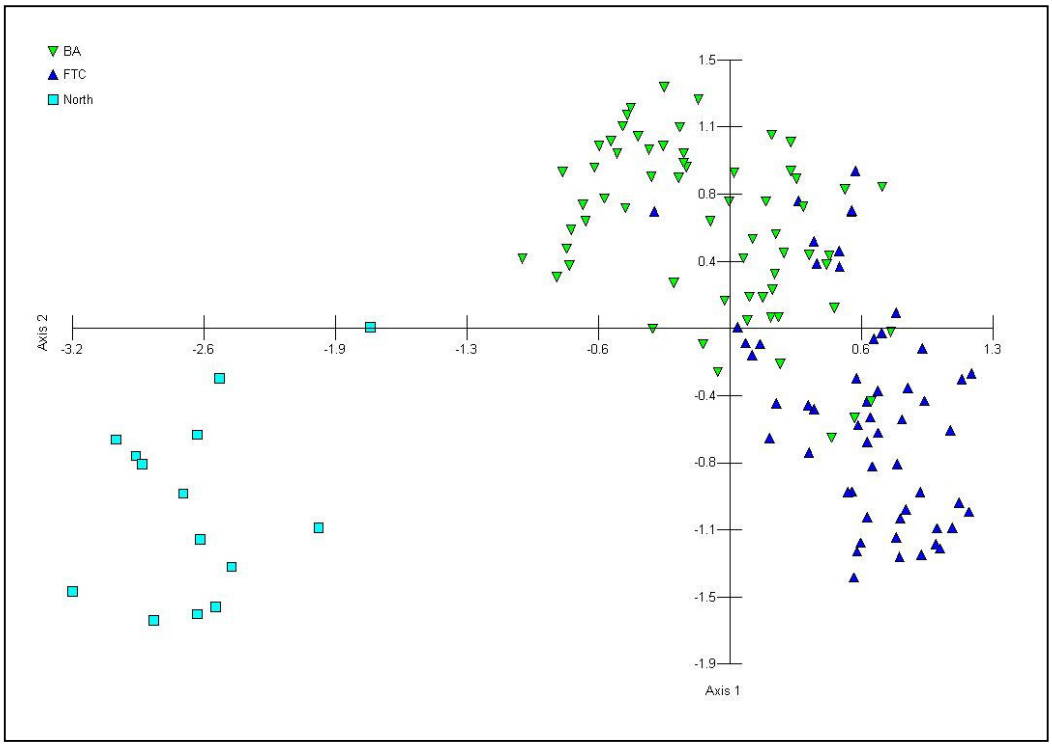
6.3.4 Genetic seed analysis

All the 11 embryos studied showed the absence of at least one of the alleles present in each locus in the putative mother tree (matrix in Appendix 9.23). The presence of an allele shared by all embryos was observed only for 3 of the 11 loci assessed (Gk-6-1, Gk-39 and CS8). Only 2 pairs of embryos (I-J and D-K) shared at least 1 allele for every locus.

6.3.5 Population analysis

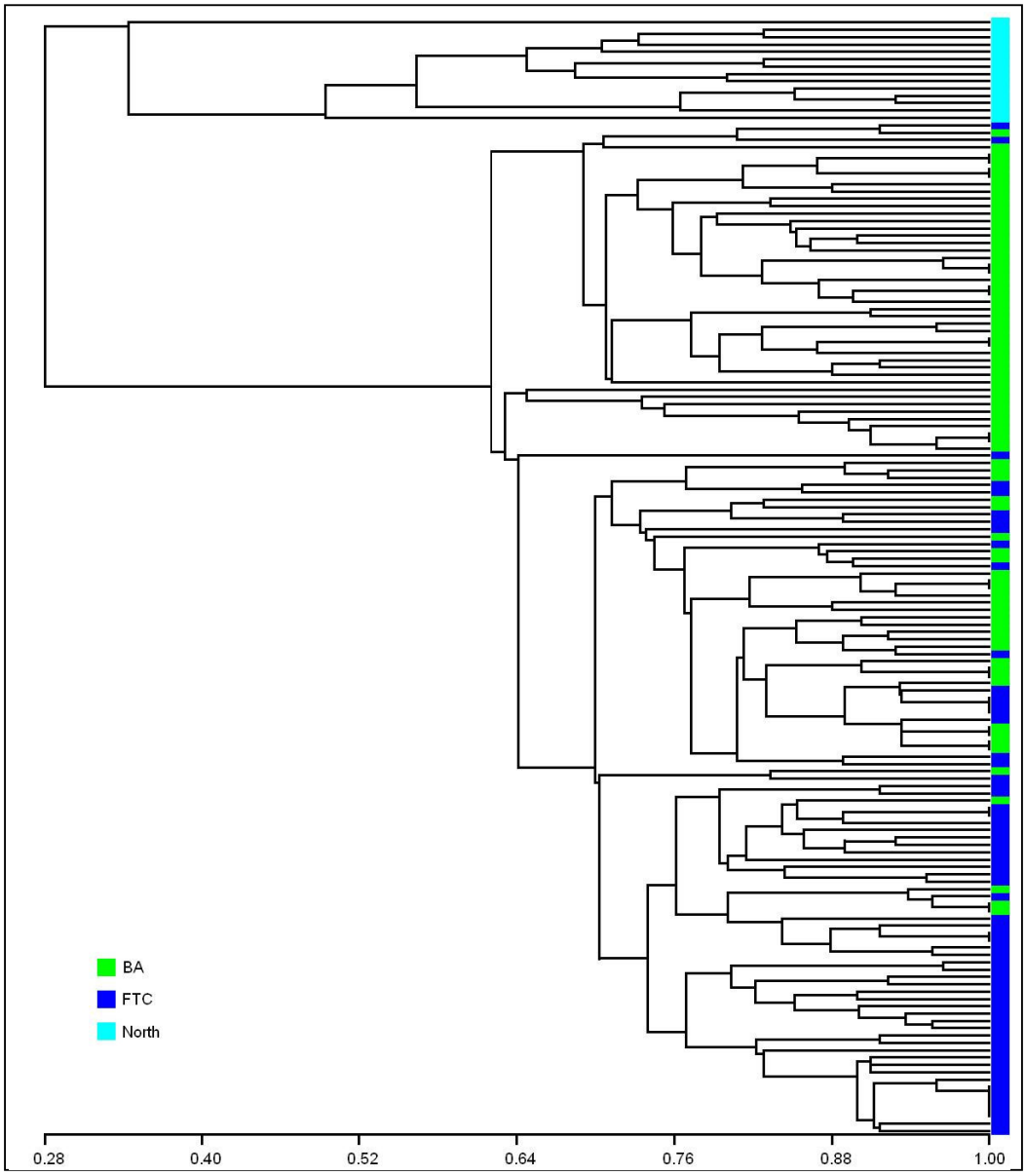
The visualisation of the genetic relationships amongst the individuals sampled is presented in the PCO analysis and the dendrogram generated by cluster analysis (Figure 6.5 and Figure 6.6).

Figure 6.5. Principal coordinate analysis for 3 populations of *Gomortega keule* using microsatellites for 9 loci.



The groups Bosques Arauco (BA, n=75) and Forestal Tierra Chilena (FTC, n=63) are the southernmost populations of the species, while the group North (populations Reserva Nacional Los Queules and Ralbún, n=14) is in the northern area of its natural distribution. The graph was generated using MVSP with the first 2 axis of eigenvalues, which accounted for 32.5% of the variance (Axis 1 19.7%, and Axis2 12.7%).

Figure 6.6. Dendrogram generated by cluster analysis for populations of *Gomortega keule*.



Populations BA (Bosques Arauco) and FTC (Forestal Tierra Chilena) are the southernmost of the species, while population North (Reserva Nacional Los Queules and Ralbún) is in the northern area of its natural distribution. The dendrogram was generated by UPGMA using the index of Nei and Li (1979) with the software MVSP.

The most frequent alleles across loci had a frequency ranging from 0.48 to 0.84 for the overall populations calculated with Powermarker. Hence, they were all polymorphic (frequency <0.95) according to Laurentin (2009). The majority of private alleles were from the population North (Table 6.4), with intra-population frequencies from 0.04 to 0.86, while the populations in the South had frequencies always <0.03 (except one allele with 0.06), which meant that private alleles were often rare in the South.

Table 6.4. Number of alleles and private alleles per locus and population.

Locus	Number of alleles				Number of private alleles		
	BA	FTC	North	Total	BA	FTC	North
Gk-1	5	4	4	7			2
Gk-6 locus 1	3	2	3	4	1		1
Gk-6 locus 2	4	4	4	6	1	1	
Gk-30	3	4	4	5			1
Gk-31	4	3	5	8	2		3
Gk-35	4	2	3	7	2		3
Gk-39	2	2	3	5			3
CS2	2	3	3	4			1
CS8	3	3	4	5			2
Mean	3.33	3.00	3.67	5.67	0.67	0.11	1.78

The largest difference between H_O and H_E (>0.2) was present in the locus Gk-31 for BA and locus Gk-35 for North (Table 6.5). The inbreeding coefficient for each locus and population showed a range of values from -0.38 to 0.86 (Table 6.6), while the highest values (F_{IS}>0.8) were observed for locus Gk-31 in BA and locus Gk-35 in North. Fixation indices (ρ_{ST} and F_{ST}) for each pair of populations are shown in Table 6.7. Exact test for HWE was significant (P<0.05) for locus Gk-31 in populations BA and FTC, Gk-35 in BA and North, and CS8 in North. Linkage disequilibrium was present in all loci although for different pairs, ranging from a single linked pair to up to 4 linked pairs within a single locus for a given population (Appendix 9.24). Two pairs of loci (Gk-6 locus 1 / Gk-6 locus 2 and Gk-39 / Gk-30) showed significant linkage disequilibrium in the 3 populations. Estimated frequencies of null alleles are presented in Table 6.8.

Table 6.5. Observed (H_O) and expected heterozygosity (H_E) for each locus and population.

Locus	BA		FTC		North		All populations	
	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E
Gk-1	0.486	0.456	0.286	0.307	0.786	0.643	0.437	0.495
Gk-6-1	0.472	0.432	0.339	0.307	0.643	0.474	0.437	0.532
Gk-6-2	0.597	0.563	0.357	0.325	0.429	0.690	0.486	0.626
Gk-30	0.361	0.405	0.714	0.724	0.500	0.585	0.514	0.609
Gk-31	0.042	0.256	0.107	0.167	0.643	0.712	0.127	0.296
Gk-35	0.431	0.489	0.589	0.503	0.071	0.500	0.458	0.598
Gk-39	0.458	0.475	0.375	0.350	0.286	0.262	0.408	0.531
CS2	0.493	0.500	0.518	0.522	0.571	0.648	0.511	0.565
CS8	0.606	0.583	0.429	0.414	0.429	0.558	0.518	0.527
Mean	0.438	0.462	0.413	0.402	0.484	0.564	0.433	0.531

Table 6.6. Inbreeding coefficient (F_{IS}) for each locus and population.

Locus	BA	FTC	North	All
Gk-1	-0.067	0.069	-0.233	-0.053
Gk-6-1	-0.095	-0.105	-0.377	-0.131
Gk-6-2	-0.061	-0.100	0.388	-0.009
Gk-30	0.109	0.014	0.150	0.063
Gk-31	0.838	0.359	0.100	0.524
Gk-35	0.120	-0.173	0.862	0.077
Gk-39	0.036	-0.072	-0.095	-0.008
CS2	0.014	0.008	0.122	0.024
CS8	-0.038	-0.035	0.239	-0.008
All	0.052	-0.026	0.145	0.035

F_{IS} Indicates deficit (>0) or excess (<0) of heterozygotes.

Table 6.7. Fixation index for population pairwise comparision.

Population pairwise	ρ_{ST}	F_{ST}
BA - FTC	0.179	0.177
BA - North	0.852	0.790
FTC - North	0.868	0.530

ρ_{ST} was caulated using Genepop (procedure by Michalakis and Excoffier 1996) and F_{ST} with Arlequin (procedure by Slatkin 1995).

Table 6.8. Estimation of null allele frequencies for each locus and population.

Locus	BA		FTC		North	
	$null_{PIM}$	$null_{IIM}$	$null_{PIM}$	$null_{IIM}$	$null_{PIM}$	$null_{IIM}$
Gk-1	0.0001	0.0323	0.0210	0.0620	0.0000	0.0530
Gk-6 locus 1	0.0000	0.0328	0.0000	0.0420	0.0000	0.0640
Gk-6 locus 2	0.0000	0.0250	0.0000	0.0350	0.1350	0.1680
Gk-30	0.0159	0.0518	0.0020	0.0370	0.0220	0.1130
Gk-31	0.2100**	0.2158**	0.0950	0.1160	0.0000	0.0850
Gk-35	0.0484	0.0638	0.0000	0.0320	0.2760**	0.2970*
Gk-39	0.0097	0.0481	0.0000	0.0440	0.0000	0.1080
CS2	0.0027	0.0449	0.0000	0.0480	0.0160	0.1030
CS8	0.0000	0.0273	0.0000	0.0410	0.1200	0.1580

Null allele frequencies were estimated using the population inbreeding model (PIM) and the individual inbreeding model (IIM) with INEst (Chybicki and Burczyk 2009). Frequencies significantly different from zero are indicated by asterisks (*, P< 0.01 and **, P<0.001).

6.4 Discussion

The yield of DNA from different samples seemed to be highly influenced by the type of tissue (actively growing, old and oxidised, dead), amount of tissue and grinding procedure. Although without an extensive evaluation, the method by Lander *et al*. (2007) gave the most consistent results, especially for dried samples.

The fact that samples from dried tissues amplified unreliably for microsatellites Gk-5 and Gk-44, could be attributed to the lower concentration and some degree of degradation of the DNA in dried tissues, since samples from embryos and *in vitro* material, which in general had good quality (undegraded DNA) and a higher quantity, amplified well. For future studies, this situation may be improved with the optimization of the $MgCl_2$ concentration in the reaction mixture and quickly dehydrating samples during field collection.

The amplification of 2 loci by the primer pair Gk-6 was not found by Lander *et al*. (2007) in the 20 individuals that they analysed. However, Baldoni et al (2009) reported that 6 of 37 microsatellites they assessed in olive amplified for 2 or more loci. Ying *et al*. (2009) also mentioned the occurrence of a primer pair which amplified for 3 loci in avocado.

In the population BA, the locus Gk-31 presented an estimated frequency of null allele significantly higher than zero (Table 6.8). For population FTC, the values were much smaller, and within its range, the highest also occured in the locus Gk-31, although with no significance. For population North, the frequency of null allele was only significant for locus Gk-35. The presence of null alleles can be considered of low importance and, although it can have some influence on the accuracy of the statistical analyses, it was not regarded as invalid.

6.4.1 Clonal genetic fidelity

No genetic difference was detected with microsatellites for each of the clones studied, suggesting that in *G. keule* there is no genetic variation or mutation, detectable by microsatellites, due to culture involving somatic embryogenesis. Low levels of somaclonal variation are unlikely to be detected by microsatellites, making this class of markers suitable for tracing clonal lines and quality control.

Similar findings have been reported using microsatellites to assess genetic variation in plant material derived from embryogenic cultures, where no genetic variation was found in trees including *Olea spp*. (Lopes *et al*. 2009), *Picea abies* (Harvengt *et al*. 2001) or *Quercus suber* (Santos *et al*. 2007). In *Betula pendula* (Ryynänen and Aronen 2005), *Quercus robur* (Valladares *et al*. 2006) and in the herbs *Swertia spp*. (Chaudhuri *et al*. 2007) and *Chlorophytum arundinaceum* (Lattoo *et al*. 2006), genetic variation was not detected by RAPD. Genetic variation was not present in *Swertia spp*. using ISSR (Joshi and Dhawan 2007).

In contrast, Marum *et al*. (2009) observed around 10% genetic variation using microsatellites in plants regenerated from somatic embryos of *Pinus pinaster*. Similar or lower levels of genetic polymorphism were reported with the use of ISSR in *Robinia ambigua* (Guo *et al*. 2006) and *Platanus acerifolia* (Huang *et al*. 2009). With the use of RAPD in *Cedrus spp*. (Renau-Morata *et al*. 2005), shoots cultured for 1 year showed low genetic variation, while the use of variable number tandemly repeats (VNTR) in *Hypericum perforatum* (Urbanová *et al*. 2006) detected minor changes after cryostorage, but not for cultured material not subjected to cryopreservation.

6.4.2 Seed tissues and parentage

All storage tissues had the same genetic profile as their respective embryo, suggesting this tissue corresponds to the endosperm. The inner storage tissue yielded no DNA, so its identity and origin remains uncertain. Further studies would need to address the anatomy of the inner storage tissue, which is an interesting subject because of the unique structure (bearing 2 different storage tissues) of the seed of *G. keule* (Dr. W Stuppy, Royal Botanic Gardens, Kew - Wakehurst Place, personal communication).

The presence of multiple alleles amongst the 11 embryos and the putative mother tree did not allow the establishment of the putative mother haplotype. The fact that all embryos showed an absence of at least 1 allele per locus of the putative mother tree, suggests that none of the embryos belonged to fruits produced by that tree. Moreover, amongst the embryos, only two pairs of embryos shared at least 1 allele from each loci, suggesting they might be siblings (embryos D / K and I / J, Appendix 9.23), but all the remaining 7 embryos would belong to the offspring of 7 different trees. Most probably, the fruits were mixed during the collection from the forest floor, or during the handling before their arrival in the laboratory.

6.4.3 Population study

The PCO analysis (Figure 6.5) showed a differentiation amongst the 3 populations which is consistent with their geographical distance. Population North is *ca.* 200 km north of populations BA and FTC, while the latter two are 7.5 km apart. The dendrogram (Figure 6.6) shows a situation consistent to the PCO analysis. A separate cluster groups all samples from the North population, while samples from the South are generally grouped after the 2 populations, although some samples from both populations shared their presence in a common branch. The PCO analysis showed a clearer and more separated position of the populations than that found by García-Gonzáles *et al*. (2008), who included 11 populations but fewer individuals per population, specifically 29 for BA and 7 for FTC.

Some of the samples that were found to be genetically identical in the dendrogram are clearly shoots of an old tree that has disappeared, confirming the importance of vegetative propagation in *G. keule*, as mentioned in previous studies (San Martín and Donoso 1996; Díaz 1999; Villegas *et al.* 2003). However, other samples that were not at such close distances may present the same profile because of a poor discrimination power by the limited number of

microsatellites used. In fact, some of the individuals differred by the presence of only one allele. Further study of those samples with improved resolution might be achieved, including more microsatellites to discriminate, or confirm as genetically identical, the dubious samples. In the case of trees growing at short distances (less than 2 m) that were found to be genetically different, the assumption of 2 or more shoots or trees growing close are part of the same old individual must be regarded with caution. The intricate way the trees occupy the space in the forest floor is also well illustrated by the case of a root (10 cm diameter) sampled at FTC and found to be different to 3 individuals growing less than 7 m apart, but identical to a large tree growing 15 m away.

Private alleles were found for all loci and especially for population North (Table 6.4). The occurrence of private alleles could be associated to the presence of rare alleles, as many of the private alleles showed low frequencies. This may partially explain the low occurrence of private alleles found in *G. keule* by García-Gonzáles *et al*. (2008), who sampled a lower number of individuals per population (7-42) across 11 populations using ISSR. A case similar to that of *G. keule*, with private alleles in small populations, was reported by Torres-Díaz *et al*. (2007) in the endangered tree *Nothofagus alessandrii*, which inhabits the same area.

Looking at heterozygosity in populations BA and FTC (Table 6.5), the biggest difference between observed and expected heterozygosity (H_E-H_O>0.2) was for locus Gk-31 in population BA, suggesting a deficit of heterozygotes. For population North, there were positive and negative values of variable magnitudes for this difference and they did not show a clear trend, possibly influenced by the rather small number of samples in this group. The high values for F_{IS} found for locus Gk-31 for BA and locus Gk-35 for North population would mean heterozygote deficit, but these figures are probably influenced by the possible presence of null alleles. The values of heterozygosity found in *G. keule* are often lower than those reported for other endangered trees (Setsuko *et al*. 2007; Tamaki *et al*. 2008) or plants (Mameli *et al*. 2008). This can be attributed to the generally smaller number of alleles per locus shown by *G. keule*.

Departures from HWE were found in a few cases. For population BA, the loci Gk-31 and Gk-35 were not in HWE, while for population FTC only the locus GK-31 was not in HWE. Locus Gk-35 was also not in HWE for population North. Lander et al (2007) only found deviation from HWE for locus Gk-39. For the locus CS8, its departure from HWE was only present in population North, but this might be influenced by the small number of samples from this population. Linkage disequilibrium was significant for some loci pairs depending on the population and was present in all 3 populations only for the pairs Gk-6 locus 1 / Gk-6 locus 2

and Gk-39 / Gk-30. Lander et al (2007) found no significance for linkage disequilibrium. In the endangered species *Centaurea horrida*, a pronounced linkage disequilibrium was observed in most of the loci studied by Mameli *et al*. (2008) using microsatellites. The latter authors mentioned that linkage disequilibrium can arise as a consequence of a reduction in effective population size that enhances genetic drift. In the present study on *G keule*, the apparent lack of a clear trend in F_{IS}, HWE deviation and linkage disequilibrium could all be due to the effect of populations being founded by a relatively small number of individuals or populations that have been isolated for a long period of time, leading to the fixation of some alleles and the prevalence of certain haplotypes.

The genetic differentiation among the populations studied was high (Table 6.7). According to Yeh (2000), F_{ST} values from 0.151 to 0.250 mean large genetic differentiation, which was found between the populations BA and FTC, while F_{ST} >0.25 means very great genetic differentiation, that was the case between the population North and BA or FTC. This situation is consistent with the PCO analysis and the geographical distance among the populations. Particularly interesting is the differentiation between populations BA and FTC, which are only 7.5 km apart.

Genetic diversity was found to be less among populations for *Fitzroya cupressoides* (Premoli *et al* 2000), *Pilgerodendron uviferum* (Allnutt *et al*. 2003) and the monkey puzzle tree *Araucaria araucana* (Bekessy *et al*. 2002), all Chilean gymnosperms with a considerably wider geographic distribution than that of *G. keule*. Accordingly, values for the fixation index assessed with microsatellites in other endangered species of trees like *Magnolia stellata* (Tamaki *et al*. 2008; Setsuko *et al*. 2007) and *Tectona grandis* (Fofana *et al*. 2009) or the shrub *Centaurea horrida* (Mameli *et al*. 2008) are surprisingly lower than those seen in *G. keule*, stressing the high differentiation in the populations of the latter tree. Genetic diversity was also important, although not as high as for *G. keule*, for the tree *Nothofagus alessandrii*, occurring in the same area and with a similar pattern of small isolated populations as in *G. keule* (Torres-Díaz *et al*. 2007).

The high differentiation between the geographically close populations BA and FTC, together with the lack of evidence of marked inbreeding, the absence of sexual regeneration at present and the apparently long regeneration cycle of *G. keule*, suggest that these populations might have become isolated long before the anthropic disturbance process begun in the area around 200 years ago (Arroyo *et al*. 2005). A population differentiation dating back to glacial times, before human-mediated fragmentation in the area, was discussed for the endemic tree *Nothofagus alessandrii* by Torres-Díaz *et al*. (2007). This contradicts the ideas of García-

Gonzáles *et al*. (2008), who proposed an active gene flow in *G. keule* until human activities would have fragmented the populations, and may have important implications in strategies for conservation management. If the genetic characteristics of the natural populations are to be conserved, it does not seem adequate to establish a pollination programme among populations, as mentioned by (Herrera *et al*. 2005). However, that also means that each population has an important amount of unique genetic information not found elswhere, and therefore they must be conserved as valuable sources of germplasm. For *ex situ* conservation, seeds sampled from as many populations as possible would capture a higher amount of genetic diversity than samples from only a few populations.

It is worth while to remark that populations BA and FTC showed to be clearly different to the North population, and this has to be taken into account for conservation purposes. The populations in the south are highly genetically different and they hold diverse and unique genetic information compared to the North population. Therefore, the conservation of these isolated populations of *G. keule* is very important.

6.5 Conclusions

Genetic variation in two embryogenic genotypes cultured *in vitro* was not detected with the use of microsatellites, suggesting that *in vitro* cultures of *G. keule* are relatively genetically stable and that this marker type could be used for tracing and quality control in clonal propagation of this species.

The genetic composition of the external storage tissue in the seed of *G. keule*, being identical to the embryo, showed that this corresponds to the endosperm.

The results support the idea that vegetative propagation is important in the natural populations studied, with some close individuals showing the same genetic profile. The populations showed large genetic differentiation, especially the populations in the south in relation to those in the north. This indicates that conservation efforts must include those southern populations, and probably other isolated populations, if an adequate extent of the genetic pool is to be preserved. Regarding agricultural and forestry potential of the species, those groups are also very important, since each isolated population keeps valuable genetic resources for the future domestication and genetic breeding of the species.

The use of microsatellites to assess genetic fidelity in tissue culture material and to characterize natural populations of *G. keule*, which are reservoirs of germplasm resources, is a useful technique allowing researchers to explore both genetic differences among groups and variation within the population.

7 General Discussion

The endangered species *Gomortega keule* has not been systematically cultivated despite its potential uses. This may be explained by the scarcity of trees that exist in natural populations and also because of their difficult propagation. Additionally, knowledge of the species at present is rather superficial and there is a clear need for detailed and systematic investigations to rectify this situation. The programme of work described in this thesis was an attempt in this direction.

The *in vitro* procedures explored in this study should facilitate propagation of the plant, providing a basis for further conservation programmes and the development of *G. keule* as a fruit crop of economic potential. Conservation may comprise the re-introduction of plants into their natural habitats and the storage of material in repositories such as cryobanks and botanic gardens. Domestication of the species will require effective propagation methods for selected genotypes and long-term effort for the development of agronomic management and possible genetic improvement through conventional breeding and somatic cell approaches.

Some of the basic techniques explored in this research permitted observations of plant development, including shoot proliferation, root induction, somatic embryogenesis and acclimatization of regenerated plants to glasshouse conditions. The establishment of cultures of *G. keule* faced the problem of contamination by micro-organisms, which is one of the major constraints for plant cell culture (Kulkarni *et al*. 2007; Leifert and Cassells 2001). The stabilization of the cultured shoots (as defined by McCown 2000) was a second problem, generally taking more than one year, and for some genotypes it was not overcome.

The amount of plant material available for experimentation was limited because of the logistics of collecting living samples from the wild. In addition, living material available from sources such as botanic gardens was in extremely short supply. In fact, it was clear that this tree hardly exists outside its natural habitat, and even well established institutions have no living specimens (Royal Botanic Gardens, Kew, UK; Botanic Garden and Botanic Museum Berlin – Dahlem, Germany) or very restricted material (The Royal Botanic Gardens, Edinburgh, UK). In the same context, it is important to further develop procedures to establish and propagate material originated from mature trees with selected characteristics. This has been the focus for previous work in trees (Sanjaya *et al*. 2006; Valladares *et al*. 2006; Seth *et*

al. 2007). Clearly, in the future there is a priority to develop a systematic collection programme with accurate documentation of elite germplasm.

Based on *in vitro* shoot proliferation and the induction of root development, a method was developed for the production of plants suitable for establishment in the field. Tissue culture in liquid media was achieved for explants that originated from zygotic embryos and from field-collected shoots. These cultures in liquid media, coupled with the use of embryogenic material, will permit the development of methods for the mass production of plants. This strategy has been investigated in several woody species of commercial interest (Yoeup and Chakrabarty 2003). The induction of somatic embryogenesis from shoots of selected genotypes of *G. keule* needs to be refined and addressed with new effort to obtain fully regenerated plants.

The ability to recover embryogenic material from cryostorage constitutes an important stage in the development of long-term conservation strategies for germplasm of *G. keule*. This is also considered important as a source of suitable material for genetic transformation in *Vitis spp*. (González-Benito et al. 2009). As the cryopreservation of shoots tips was not possible in *G. keule*, the induction of somatic embryogenesis using material from elite trees will be of considerable importance. The regeneration, acclimatization and evaluation of the stability (either genetic as well as phenotypic) of plants obtained after cryopreservation or embryogenic cultures must be addressed in the future. Slow growing cultures of shoot tips can be explored as an alternative means for germplasm conservation, especially for situations where selected genotypes do not undergo somatic embryogenesis. This strategy is currently in use for other threatened species (Paunescu 2009) and in the case of *G. keule*, it will have the advantage of preserving considerable genetic variation with the storage of material originated from seeds of different populations. The conservation of orchid seeds has been discussed as an effective means to maintain genetic diversity (Sarasan *et al*. 2006). It may be expected that the storage of such material in *G. keule* will capture a diverse group of genotypes, representing the genetic diversity of the species in a more efficient way than cloning natural trees, which in turn is difficult and still uncertain.

One of the main concerns for plants that originate *in vitro* is somaclonal variation (Phillips *et al*. 1994). This was evaluated in the present investigation using cytological observations and microsatellite analysis. Cytological observations of chromosomes were hampered by the scarcity of material and the very small size of the chromosomes, making it impossible to count chromosome numbers with confidence and to record accurately their morphology, in accordance with previous reports that discussed the constraints of this technique (Rani and

Raina 2000; Harding 2004). The use of microsatellites to assess somaclonal variation has been reported in several tree species (Harvengt *et al*. 2001; Santos *et al*. 2007; Lopes *et al*. 2009; Marum *et al*. 2009), and in the present study it was regarded as the preferred option for *G. keule*. The evaluation of cultured material using microsatellites showed no genetic variation, suggesting that the proliferation of somatic embryos is genetically stable. However, future studies are needed, including assessments of the phenotype of regenerated and acclimatized plants in the field, since a multidisciplinary approach would provide the best characterization of tissue culture-derived plants (Rani and Raina 2000).

The use of microsatellites to study natural populations showed pronounced differentiation amongst the latter, indicating that they are genetically isolated. Regarding *in situ* conservation, this finding highlighted the need of small populations to be protected. The germplasm contained in those small and isolated populations is also important for future breeding programs, because they may contain traits of agronomic interest that may be absent in other places.

The present work provides feasible methods for the production of plants of *G. keule* using *in vitro* techniques. This responds to the difficult propagation of the species and constitutes a significant step towards its domestication. Those methods, together with the procedures developed for cryopreservation, will permit *ex situ* conservation initiatives. Additional procedures explored, including the induction and proliferation of somatic embryos as well as the use of cultures in liquid medium, will enable further research that may lead to the development of methods for the mass production of plants and genetic transformation for plant breeding or research purposes. The use of microsatellites seemed to be a reliable methodology for detecting somaclonal variation, while its application to natural populations permitted to assess the genetic structure and relationship within and amongst groups of trees, providing useful information that may assist the design of conservation strategies and for studies of the natural history of *G. keule*.

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9 Appendices

9.1 Media used for shoot cultures of *Gomortega keule*.

Medium code [1]	GA_3 (mg/l)	Auxin (mg/l)	Cytokinin (mg/l)	Sucrose (g/l)	PVP (g/l)	AC (g/l)	Agar (g/l)
MS20	0.5	NAA 0.1	BAP 1.0	30	10	-	8
WP21	0.5	NAA 0.1	BAP 1.0	30	10	-	6
MS24	0.5	IBA 1.0	BAP 1.0	30	10	-	8
WP27	0.5	IBA 1.0	BAP 1.0	30	10	-	6
WP28	0.5	IBA 2.0	BAP 1.0	20	10	-	6
WP31	0.5	NAA 0.1	BAP 1.0	20	10	-	6
WP32	-	NAA 0.1	BAP 1.0	30	10	-	6
WP34	-	IBA 1.0	-	20	10	2	6
WP35	-	IBA 5.0	-	20	10	2	6
WP40	-	NAA 0.1	BAP 1.0	20	10	-	6
WP41	1.0	NAA 0.1	BAP 1.0	20	10	-	6
WP42	5.0	NAA 0.1	BAP 1.0	20	10	-	6
WP43	0.5	IBA 1.0	BAP 1.0	20	10	-	6
WP44	0.5	IBA 5.0	BAP 1.0	20	10	-	6
WP45	0.5	NAA 0.1	BAP 1.0	20	10	-	6
WP46	0.5	NAA 0.1	BAP 1.0	20	10	2	6
WP47	0.5	NAA 0.1	TDZ 0.1	20	10	-	6
WP48	0.5	NAA 0.1	TDZ 0.4	20	10	-	6
WP52A	0.5	IBA 1.0	BAP 0.1	20	10	-	6
WP53B	0.5	IBA 1.0	BAP 1.0	20	-	1	6
WP53A	0.5	IBA 2.0	BAP 0.1	20	10	-	6
WP54A	0.5	IBA 1.0	BAP 1.0	20	-	1	8
WP55	0.5	IBA 1.0	BAP 1.0	20	10	1	8
WP56	0.5	NAA 0.1	BAP 1.0	20	10	1	8
WP57	0.5	IBA 1.0	BAP 0.1	20	10	1	8
WP59	0.5	IBA 1.0	BAP 1.0	20	10	2	8
WP60	0.5	IBA 10.0	BAP 1.0	20	10	2	8
WP61	0.5	IBA 5.0	BAP 1.0	20	10	-	6

Media used for shoot cultures of *Gomortega keule* (continued).

Medium code [1]	GA_3 (mg/l)	Auxin (mg/l)	Cytokinin (mg/l)	Sucrose (g/l)	PVP (g/l)	AC (g/l)	Agar (g/l)
WP62	0.5	IBA 5.0	BAP 5.0	20	10	-	6
WP63	0.5	IBA 10.0	BAP 1.0	20	10	-	6
WP64	0.5	IBA 1.0	BAP 5.0	20	10	-	6
WP65	0.5	NAA 0.1	BAP 1.0	20	-	-	8
WP66	0.5	NAA 0.1	BAP 1.0	20	10	-	8
WP67	0.5	NAA 0.1	BAP 1.0	20	-	0.5	8
WP68	0.5	NAA 0.1	BAP 1.0	20	10	0.5	8
WP69	0.5	2,4-D 0.1	2iP 0.1	20	10	-	6
WP70	0.5	2,4-D 1.0	2iP 1.0	20	10	-	6
WP71	0.5	NAA 0.1	2iP 0.1	20	10	-	6
WP72	0.5	NAA 0.1	2iP 1.0	20	10	-	6
WP73	0.5	2,4-D 0.1	BAP 1.0	20	10	-	6
WP74	0.5	2,4-D 1.0	BAP 1.0	20	10	-	6
WP81	0.5	NAA 0.1	BAP 1.0	20	-	-	8
WP82	0.5	NAA 0.1	BAP 1.0	20	-	1	8
WP83	0.5	NAA 0.1	BAP 1.0	20	10 [2]	-	8
WP85	0.5	NAA 0.1	BAP 1.0	20	10	-	8
WP91	-	NAA 0.1	BAP 1.0	20	-	-	8
WP92	1.0	NAA 0.1	BAP 1.0	20	-	-	8
WP93	5.0	NAA 0.1	BAP 1.0	20	-	-	8
WP94	10.0	NAA 0.1	BAP 1.0	20	-	-	8
WP95	-	-	-	20	-	-	8
WP96	-	-	-	20	-	1	8
WP97	-	-	-	20	-	2	8
WP99	0.5	NAA 0.1	2iP 0.1	20	-	2	8
WP100A	0.5	NAA 0.1	2iP 1.0	20	-	-	8
WP100B	-	NAA 0.1	Kin 0.1	20	-	-	8
WP101	-	NAA 0.1	Ztn 0.1	20	-	-	8
WP102	-	NAA 0.1	Kin 1.0	20	-	-	8
WP103	-	NAA 0.1	Ztn 1.0	20	-	-	8

[1] 'MS' corresponded to MS basal medium and 'WP' to WP basal medium.

[2] PVP 10,000 was used.

9.2 Effect of basal media on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Variable average value	SD	Number of replicates	Statistical test used	P value
C	08/02/2008	101	Weight gained (g)	MS20	0.13	0.07	6	Mann-Whitney	0.5745
				WP21	0.18	0.23	6		
			New shoots per initial shoot (number)	MS20	2.67	1.03	6	Mann-Whitney	0.7384
				WP21	4.67	5.16	6		
			Shoot height (mm)	MS20	10.67	1.21	6	t Test	0.2180
				WP21	9.50	1.76	6		
			Shoot width (mm)	MS20	16.83	3.60	6	t Test	0.7800
				WP21	17.67	6.09	6		
S4-E1	28/01/2008	91	Weight gained (g)	MS20	0.73	1.03	12	Mann-Whitney	0.7949
				WP21	0.70	0.60	12		
			New shoots per initial shoot (number)	MS20	8.63	15.41	6	Mann-Whitney	0.8545
				WP21	3.30	1.72	5		
			Shoot height (mm)	MS20	15.83	6.45	12	t Test	0.7860
				WP21	15.15	5.89	13		
			Shoot width (mm)	MS20	26.67	6.14	12	t Test	0.2800
				WP21	24.00	5.89	13		

Effect of basal media on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-F16	07/02/2008	104	Weight gained (g)	MS20	0.38	0.53	11	Mann-Whitney	0.4050
				WP21	0.51	0.65	12		
			New shoots per initial shoot (number)	MS20	1.75	1.75	12	Mann-Whitney	0.5270
				WP21	1.94	1.94	12		
			Shoot height (mm)	MS20	13.17	4.84	12	t Test	0.4030
				WP21	15.00	5.66	12		
S3-214	23/01/2008	90	Weight gained (g)	MS20	0.27	0.48	9	Mann-Whitney	0.8194
				WP21	0.43	0.64	11		
			New shoots per initial shoot (number)	MS20	1.95	1.62	11	Mann-Whitney	0.2600
				WP21	2.80	1.87	10		
			Explant height (mm)	MS20	9.50	5.40	12	t Test	0.7860
				WP21	12.42	7.17	12		
			Explant diameter (mm)	MS20	17.83	7.38	12	t Test	0.2740
				WP21	18.75	8.87	12		

Effect of basal media on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-B6	24/01/2008	86	Weight gained (g)	MS20	1.10	0.97	18	t Test	0.4860
				WP21	1.32	0.79	13		
			New shoots per initial shoot (number)	MS20	4.50	2.50	9	Mann-Whitney	0.1051
				WP21	8.55	5.67	11		
			Explant height (mm)	MS20	14.53	3.36	19	t Test	0.3430
				WP21	15.85	4.45	13		
			Explant diameter (mm)	MS20	24.58	6.64	19	t Test	0.3750
				WP21	27.15	7.85	13		
S4-A3	26/02/2008	92	Final weight (g)	MS20	0.69	0.42	14	Mann-Whitney	0.0007 **
				WP21	2.16	0.83	7		
			New shoots per initial shoot (number)	MS20	5.50	2.47	14	Mann-Whitney	0.0005 **
				WP21	15.00	4.08	7		
			Explant height (mm)	MS20	15.14	3.25	14	t Test	0.0010 **
				WP21	21.86	3.34	7		
			Explant diameter (mm)	MS20	24.71	6.02	14	t Test	0.0030 **
				WP21	35.29	5.99	7		

Effect of basal media on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Variable average value	SD	Number of replicates	Statistical test used	P value
S3-60	15/02/2008	47	Weight gained (g)	MS20	0.25	0.29	3	Mann-Whitney	0.2330
				WP21	0.54	0.55	5		
			New shoots per initial shoot (number)	MS20	1.67	0.58	3	Mann-Whitney	0.1122
				WP21	5.40	5.50	5		
			Explant height (mm)	MS20	9.75	4.86	4	t Test	0.6260
				WP21	11.40	4.72	5		
			Explant diameter (mm)	MS20	17.75	5.38	4	t Test	0.2300
				WP21	22.60	5.46	5		
S3-111	30/05/2008	121	New shoots per initial shoot (number)	MS20	2.57	0.82	4	Mann-Whitney	0.8839
				WP21	3.03	0.99	4		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.3 Effect of sucrose concentration on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Sucrose in medium (g/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
C	08/02/2008	101	Weight gained (g)	WP 21	30	0.18	0.23	6	Mann-Whitney	0.1705
				WP 31	20	0.05	0.05	6		
			New shoots per initial shoot (number)	WP 21	30	4.67	5.16	6	Mann-Whitney	0.0480 *
				WP 31	20	1.42	0.92	6		
			Explant height (mm)	WP 21	30	9.50	1.76	6	Mann-Whitney	0.8030
				WP 31	20	10.67	3.93	6		
			Explant diameter (mm)	WP 21	30	17.67	6.09	6	t Test	0.0670
				WP 31	20	11.17	4.62	6		
S4-E1	28/01/2008	91	Weight gained (g)	WP 21	30	0.70	0.60	12	t Test	0.8190
				WP 31	20	0.61	0.42	10		
			New shoots per initial shoot (number)	WP 21	30	3.30	1.72	5	Mann-Whitney	0.2909
				WP 31	20	1.93	0.51	3		
			Explant height (mm)	WP 21	30	15.15	5.89	13	t Test	0.8490
				WP 31	20	14.18	5.46	11		
			Explant diameter (mm)	WP 21	30	24.00	5.89	13	t Test	0.6790
				WP 31	20	24.45	5.66	11		

Effect of sucrose concentration on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Sucrose in medium (g/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-F16	07/02/2008	104	Weight gained (g)	WP 21	30	0.51	0.65	12	Mann-Whitney	0.4785
				WP 31	20	0.65	0.66	11		
			New shoots per initial shoot (number)	WP 21	30	1.94	1.94	12	Mann-Whitney	0.7994
				WP 31	20	1.86	1.86	11		
			Explant height (mm)	WP 21	30	15.00	5.66	12	t Test	0.4590
				WP 31	20	17.00	6.91	11		
S4-B6	24/01/2008	86	Weight gained (g)	WP 21	30	1.32	0.79	13	t Test	0.5810
				WP 31	20	1.47	0.67	20		
			New shoots per initial shoot (number)	WP 21	30	8.55	5.67	11	Mann-Whitney	0.5086
				WP 31	20	6.50	3.84	11		
			Explant height (mm)	WP 21	30	15.85	4.45	13	t Test	0.8920
				WP 31	20	16.05	3.65	20		
			Explant diameter (mm)	WP 21	30	27.15	7.85	13	Mann-Whitney	0.5511
				WP 31	20	29.50	4.73	20		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.4 Effect of agar concentration on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Agar content in medium (g/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-A3	14/04/2008	47	Final weight (g)	WP53B	6	0.12	0.10	12	Mann-Whitney	0.8850
				WP54A	8	0.14	0.20	12		
			Weight gained (g)	WP53B	6	0.03	0.10	12	Mann-Whitney	0.8620
				WP54A	8	0.04	0.16	12		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.5 Effect of temperature on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Temperature (ºC)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-A3	09/09/2008	82	Shoots per explant (number)	15	6.31	2.69	14	Kruskal-Wallis	0.0470 *
				18	5.16	2.90	15		
				25	3.82	2.28	14		
S4-A3	14/11/2008	66	Shoots per explant (number)	15	2.64	0.88	12	Kruskal-Wallis	0.1550
				18	2.72	1.82	12		
				25	1.91	0.70	11		
S4-E1	14/11/2008	64	Shoots per explant (number)	15	3.50	1.71	8	ANOVA	0.2770
				18	4.13	1.36	8		
				25	2.78	1.79	8		
S4-F16	14/11/2008	64	Shoots per explant (number)	15	1.78	0.56	8	ANOVA	0.2170
				18	2.34	0.46	8		
				25	2.09	1.03	8		
C	21/01/2009	61	Shoots per explant (number)	18	2.85	1.32	12	t Test	0.3710
				25	2.35	0.64	12		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.6 Effect of high frequency subculture (HFS) on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Treatment	Variable average value	SD	Number of replicates	Statistical test used	P value
C	12/04/2008	57	Weight gained (g)	Control	0.12	0.09	12	t Test	0.0050 **
				HFS	0.26	0.13	12		
			Explant height (mm)	Control	7.50	3.87	12	t Test	0.0570
				HFS	10.17	2.37	12		
			Explant diameter (mm)	Control	12.42	5.92	12	t Test	0.0590
				HFS	16.75	4.61	12		
S4-F16	14/05/2008	96	Final weight (g)	Control	0.49	0.67	12	Mann-Whitney	0.0010 **
				HFS	3.13	2.56	11		
			Shoots per explant (number)	Control	3.00	2.22	12	Mann-Whitney	0.0568
				HFS	4.36	1.91	11		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.7 Effect of different auxins (NAA or IBA) on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Auxin in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
C	08/02/2008	101	Weight gained (g)	WP28	IBA (2.0)	0.28	0.40	3	Mann-Whitney	0.5151
				WP31	NAA (0.1)	0.05	0.05	6		
			New shoots per initial shoot (number)	WP28	IBA (2.0)	1.33	0.29	3	Mann-Whitney	0.7860
				WP31	NAA (0.1)	1.42	0.92	6		
			Explant height (mm)	WP28	IBA (2.0)	10.67	4.04	3	t Test	1.0000
				WP31	NAA (0.1)	10.67	3.93	6		
			Explant diameter (mm)	WP28	IBA (2.0)	17.00	2.00	3	t Test	0.0390 *
				WP31	NAA (0.1)	11.17	4.62	6		
S4-B6	24/01/2008	86	Weight gained (g)	MS 20	NAA (0.1)	1.10	0.97	18	t Test	0.0600
				MS 24	IBA (1.0)	1.81	0.66	4		
			Explant height (mm)	MS 20	NAA (0.1)	14.53	3.36	19	t Test	0.5810
				MS 24	IBA (1.0)	15.75	3.77	4		
			Explant diameter (mm)	MS 20	NAA (0.1)	24.58	6.64	19	t Test	0.4670
				MS 24	IBA (1.0)	27.25	5.91	4		
S4-A3	14/04/2008	47	Final weight (g)	WP55	IBA (1.0)	0.06	0.04	12	t Test	0.6900
				WP56	NAA (0.1)	0.07	0.05	12		

Effect of different auxins (NAA or IBA) on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Auxin in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-E11	22/02/2008	85	Weight gained (g)	WP21	NAA (0.1)	0.62	0.72	6	t Test	0.7530
				WP27	IBA (1.0)	0.74	0.72	8		
			New shoots per initial shoot (number)	WP21	NAA (0.1)	1.70	0.84	5	t Test	0.3420
				WP27	IBA (1.0)	2.42	1.43	6		
S4-F16	14/05/2008	96	Weight gained (g)	WP31	NAA (0.1)	0.43	0.64	12	Mann-Whitney	0.1653
				WP43	IBA (1.0)	0.18	0.29	11		
			Shoots per explant (number)	WP31	NAA (0.1)	3.00	2.22	12	Mann-Whitney	0.5405
				WP43	IBA (1.0)	2.64	2.20	11		
S3-60	15/02/2008	47	Weight gained (g)	WP21	NAA (0.1)	0.54	0.55	5	t Test	0.8450
				WP27	IBA (1.0)	0.49	0.23	7		
			Shoots per explant (number)	WP21	NAA (0.1)	5.40	5.50	5	Mann-Whitney	0.3551
				WP27	IBA (1.0)	5.57	3.15	7		
			Explant height (mm)	WP21	NAA (0.1)	11.40	4.72	5	t Test	0.2950
				WP27	IBA (1.0)	14.43	4.47	7		
			Explant diameter (mm)	WP21	NAA (0.1)	22.60	5.46	5	t Test	0.8800
				WP27	IBA (1.0)	22.14	4.18	7		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.8 Effect of IBA concentration on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	IBA in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
C	23/05/2008	53	New shoots per initial shoot (number)	WP61	5.0	1.80	0.84	5	Mann-Whitney	0.8345
				WP63	10.0	1.40	0.89	5		
			New shoots per initial shoot (number)	WP62	5.0	1.50	1.00	4	Mann-Whitney	1.0000
				WP64	1.0	2.00	2.00	4		
S1 A	04/03/2008	119	New shoots per initial shoot (number)	WP34	1.0	1.17	0.41	6	Mann-Whitney	1.0000
				WP35	5.0	1.33	0.82	6		
S4-D20	27/05/2008	98	Shoots per explant (number)	WP52A	1.0	1.50	2.07	6	Mann-Whitney	1.0000
				WP53A	2.0	1.50	2.35	6		
S4-F16	14/05/2008	96	Weight gained (g)	WP43	1.0	0.18	0.29	11	Mann-Whitney	0.5372
				WP44	5.0	0.16	0.13	12		
			New shoots per initial shoot (number)	WP43	1.0	2.64	2.20	11	Mann-Whitney	0.9484
				WP44	5.0	2.17	1.19	12		
S3-60	04/03/2008	96	Shoots per explant (number)	WP34	1.0	1.57	1.57	7	Mann-Whitney	0.6016
				WP35	5.0	1.86	1.86	7		
			Normal shoots per explant (number)	WP34	1.0	0.86	0.86	7	Mann-Whitney	0.6264
				WP35	5.0	0.57	0.57	7		

Effect of IBA concentration on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	IBA in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S3-60	07/05/2008	64	Explants with normal shoots (number)	WP59	1.0	0.52		23	Chi Square	0.6700
				WP60	10.0	0.50		20		
S3-60	21/05/2008	51	Shoots per explant (number)	WP61	5.0	2.11	0.93	9	Mann-Whitney	0.3165
				WP63	10.0	1.50	0.84	6		
			Shoots per explant (number)	WP62	5.0	1.83	0.41	6	Mann-Whitney	0.9055
				WP64	1.0	1.91	0.70	11		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.9 Effect of BAP concentration on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	BAP in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
C	23/05/2008	53	New shoots per initial shoot (number)	WP61	1.0	1.80	0.84	5	Mann-Whitney	0.3913
				WP62	5.0	1.50	1.00	4		
S4-A3	14/04/2008	47	Final weight (g)	WP55	1.0	0.06	0.04	12	t Test	0.7980
				WP57	0.1	0.09	0.07	12		
S3-60	21/05/2008	51	Shoots per explant (number)	WP61	1.0	2.11	0.93	9	Mann-Whitney	0.6722
				WP62	5.0	1.83	0.41	6		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.10 Effect of different auxins (NAA or 2,4-D) on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Auxin in medium (mg/l)	Observed	Total	Statistical test used	P value
S4-B6	27/05/2008	55	Explants with abundant callus on stem base (number)	WP31	NAA (0.1)	3	12	Chi Square	0.0050 **
				WP73	2,4-D (0.1)	15	15		
			Explants with abundant callus on stem base (number)	WP69	2,4-D (0.1)	14	18	Chi Square	0.0180 *
				WP71	NAA (0.1)	4	18		
			Explants with shoots elongated over 5 mm (number)	WP31	NAA (0.1)	9	12	Chi Square	0.0830
				WP73	2,4-D (0.1)	3	15		
S4-B6	27/05/2008	55	Explants with abundant callus on stem base (number)	WP73	2,4-D (0.1)	15	15	Chi Square	0.0000 **
				WP74	2,4-D (1.0)	0	12		
			Explants with callus on leaf base and or shoot apex (number)	WP73	2,4-D (0.1)	4	15	Chi Square	0.0460 *
				WP74	2,4-D (1.0)	12	12		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.11 Effect of different cytokinins (BAP, 2iP, Kin or Ztn) on tissue cultures of *Gomortega keule*. Results from continuous data.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Cytokinin in medium (mg/l)	Variable average value [1]	SD	Number of replicates	Statistical test used	P value
S4-B6	22/12/2008	137	Shoots per explant (number)	WP81	IBA (1)	7.15	0.58	5	t Test	<0.001 **
				WP100A	2iP (1)	3.17	0.54	6		
			Final weight (g)	WP81	IBA (1)	0.38	0.13	5	t Test	0.1110
				WP100A	2iP (1)	0.22	0.16	6		
S4-A3	22/12/2008	137	Shoots per explant (number)	WP81	IBA (1)	8.29	3.51	6	t Test	0.1380
				WP100A	2iP (1)	5.21	3.03	6		
			Final weight (g)	WP81	IBA (1)	0.53	0.43	6	t Test	0.9860
				WP100A	2iP (1)	0.52	0.25	6		
S4-B6	22/12/2008	111	Shoots per explant (number)	WP81	BAP (1)	6.67 a	1.05	6	ANOVA	<0.001 **
				WP102	Kin (1)	2.44 b	0.62	6		
				WP103	Ztn (1)	4.27 b	2.07	5		
			Final weight (g)	WP81	BAP (1)	0.39	0.13	6	ANOVA	0.1020
				WP102	Kin (1)	0.18	0.09	6		
				WP103	Ztn (1)	0.38	0.29	4		

Effect of different cytokinins (BAP, 2iP, Kin or Ztn) on tissue cultures of Gomortega keule. Results from continuous data (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Cytokinin in medium (mg/l)	Variable average value [1]	SD	Number of replicates	Statistical test used	P value
S4-A3	22/12/2008	111	Shoots per explant (number)	WP81	BAP (1)	5.85	2.60	5	ANOVA	0.1780
				WP102	Kin (1)	3.06	1.88	4		
				WP103	Ztn (1)	4.67	1.69	6		
			Final weight (g)	WP81	BAP (1)	0.31	0.30	5	ANOVA	0.2870
				WP102	Kin (1)	0.14	0.11	4		
				WP103	Ztn (1)	0.35	0.14	6		

[1] Letters indicate differences under Tukey's confidence intervals (95%).

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.12 Effect of different cytokinins (BAP, 2iP, Kin or Ztn) on tissue cultures of *Gomortega keule*. Results from discrete data.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Cytokinin in medium (mg/l)	Observed	Total	Statistical test used	P value
S4-B6	27/05/2008	55	Explants with abundant callus on stem base (number)	WP31	BAP (1)	3	12	Chi Square	0.0010 **
				WP72	2iP (1)	18	18		
			Explants with callus on leaf base or shoot apex (number)	WP70	2iP (1)	14	15	Chi Square	0.6950
				WP74	BAP (1)	12	12		
			Explants with shoots elongated over 5 mm (number)	WP31	BAP (1)	9	12	Chi Square	0.4390
				WP72	2iP (1)	6	18		
S4-B6	22/12/2008	137	Explants with callus (number)	WP81	BAP (1)	0	20	Chi Square	0.0010 **
				WP100A	2iP (1)	10	24		
S4-A3	22/12/2008	137	Explants with callus (number)	WP81	BAP (1)	8	24	Chi Square	0.0001 **
				WP100A	2iP (1)	24	24		
S4-B6	22/12/2008	111	Explants with callus (number)	WP81	BAP (1)	7	18	Chi Square	0.1680
				WP102	Kin (1)	5	18		
				WP103	Ztn (1)	9	15		
S4-A3	22/12/2008	111	Explants with callus (number)	WP81	BAP (1)	8	20	Chi Square	<0.001 **
				WP102	Kin (1)	14	16		
				WP103	Ztn (1)	23	24		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.13 Effect of cytokinin (2iP, Kin or Ztn) concentration on tissue cultures of *Gomortega keule*. Results from discrete data.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Cytokinin in medium (mg/l)	Observed	Total	Statistical test used	P value
S4-B6	27/05/2008	55	Explants with abundant callus on stem base (number)	WP71	2iP (0.1)	4	18	Chi Square	0.0030 **
				WP72	2iP (1.0)	18	18		
			Explants with shoots elongated over 5 mm (number)	WP71	2iP (0.1)	4	18	Chi Square	0.5270
				WP72	2iP (1.0)	6	18		
S4-B6	22/12/2008	137	Explants with callus (number)	99	2iP (0.1)	1	24	Chi Square	0.0020 **
				100A	2iP (1.0)	10	24		
S4-A3	22/12/2008	137	Explants with callus (number)	99	2iP (0.1)	20	20	-	-
				100A	2iP (1.0)	24	24		
S4-B6	22/12/2008	111	Explants with callus (number)	100B	Kin (0.1)	5	18	-	-
				102	Kin (1.0)	5	18		
			Explants with callus (number)	101	Ztn (0.1)	7	15	Chi Square	0.4642
				103	Ztn (1.0)	9	15		
S4-A3	22/12/2008	111	Explants with callus (number)	100B	Kin (0.1)	15	24	Chi Square	0.0828
				102	Kin (1.0)	14	16		
			Explants with callus (number)	101	Ztn (0.1)	18	24	Chi Square	0.0409 *
				103	Ztn (1.0)	23	24		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.14 Effect of cytokinin (2iP, Kin, Ztn or TDZ) concentration on tissue cultures of *Gomortega keule*. Results from continuous data.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Cytokinin in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
C	27/03/2008	48	Weight gained (g)	WP47	TDZ (0.1)	0.05	0.06	6	t Test	0.0870
				WP48	TDZ (0.4)	0.11	0.06	12		
S4-B6	22/12/2008	137	Shoots per explant (number)	99	2iP (0.1)	2.79	0.64	6	t Test	0.3010
				100A	2iP (1.0)	3.17	0.54	6		
			Final weight (g)	99	2iP (0.1)	0.07	0.03	6	Mann-Whitney	0.0303 *
				100A	2iP (1.0)	0.22	0.16	6		
S4-A3	22/12/2008	137	Shoots per explant (number)	99	2iP (0.1)	3.75	1.74	5	t Test	0.3480
				100A	2iP (1.0)	5.21	3.03	6		
			Final weight (g)	99	2iP (0.1)	0.30	0.13	5	t Test	0.1010
				100A	2iP (1.0)	0.52	0.25	6		

Effect of cytokinin (2iP, Kin, Ztn or TDZ) concentration on tissue cultures of *Gomortega keule*. Results from continuous data (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	Cytokinin in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-B6	22/12/2008	111	Shoots per explant (number)	100B	Kin (0.1)	2.83	1.15	6	t Test	0.4900
				102	Kin (1.0)	2.44	0.62	6		
			Final weight (g)	100B	Kin (0.1)	0.15	0.04	5	t Test	0.4050
				102	Kin (1.0)	0.18	0.09	6		
			Shoots per explant (number)	101	Ztn (0.1)	3.47	0.30	5	Mann-Whitney	0.9139
				103	Ztn (1.0)	4.27	2.07	5		
			Final weight (g)	101	Ztn (0.1)	0.22	0.11	5	t Test	0.3470
				103	Ztn (1.0)	0.38	0.29	4		
S4-A3	22/12/2008	111	Shoots per explant (number)	100B	Kin (0.1)	2.63	0.90	6	t Test	0.8640
				102	Kin (1.0)	3.06	1.88	4		
			Final weight (g)	100B	Kin (0.1)	0.09	0.05	6	t Test	0.4460
				102	Kin (1.0)	0.14	0.11	4		
			Shoots per explant (number)	101	Ztn (0.1)	1.71	1.01	6	t Test	0.0060 **
				103	Ztn (1.0)	4.67	1.69	6		
			Final weight (g)	101	Ztn (0.1)	0.08	0.07	6	t Test	0.0040 **
				103	Ztn (1.0)	0.35	0.14	6		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.15 Effect of GA_3 concentrations on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	GA_3 in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-A3	26/02/2008	92	Final weight (g)	WP21	0.5	2.16	0.83	7	t Test	0.4360
				WP32	0.0	1.80	0.61	4		
			New shoots per initial shoot (number)	WP21	0.5	15.00	4.08	7	Mann-Whitney	0.7685
				WP32	0.0	12.50	8.66	4		
			Explant height (mm)	WP21	0.5	21.86	3.34	7	t Test	0.3580
				WP32	0.0	19.75	3.40	4		
			Explant diameter (mm)	WP21	0.5	35.29	5.99	7	t Test	0.1870
				WP32	0.0	30.75	4.35	4		
S4-E1	27/05/2008	96	Shoots per explant (number)	WP40	0.0	0.92	1.08	12	Kruskal-Wallis	0.6640
				WP41	1.0	0.83	0.83	12		
				WP42	5.0	1.31	1.32	13		
			Explants with callus (number)	WP40	0.0	0.75		12	Chi Square	0.5540
				WP41	1.0	0.42		12		
				WP42	5.0	0.62		13		

Effect of GA_3 concentrations on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	GA_3 in medium (mg/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-F16	09/09/2008	78	Shoots per explant (number)	WP91	0.0	3.86	2.63	12	Kruskal-Wallis	0.3130
				WP92	1.0	2.78	1.99	12		
				WP93	5.0	2.15	1.23	12		
				WP94	10.0	3.31	2.66	12		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.16 Effect of PVP on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	PVP in medium (g/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-A3	14/04/2008	47	Final weight (g)	WP54A	0	0.14	0.20	12	Mann-Whitney	0.0319 *
				WP55	10	0.06	0.04	12		
S4-A3	23/05/2008	52	Shoots per explant (number)	WP65	0	3.13	2.07	15	Mann-Whitney	0.7156
				WP66	10	2.87	2.39	15		
			Shoots per explant (number)	WP67	0	1.59	1.00	17	Mann-Whitney	0.9850
				WP68	10	1.56	1.04	18		
			Explants with callus at the base of stem (number)	WP65 + WP67	0	0.32		33	Chi Square	0.6700
				WP66 + WP68	10	0.40		30		
S4-B6	23/05/2008	52	Shoots per explant (number)	WP65	0	1.78	1.20	9	Mann-Whitney	0.6087
				WP66	10	2.00	1.12	9		
			Shoots per explant (number)	WP67	0	0.92	0.67	12	Mann-Whitney	0.4793
				WP68	10	1.20	0.86	15		

Effect of PVP on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	PVP in medium (g/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-E1	03/09/2008	113	Shoots per explant (number)	WP81	0	4.28	1.31	13	Mann-Whitney	1.0000
				WP83 PVP10	10	4.36	1.73	13		
			Shoots per explant (number)	WP81	0	4.28	1.31	13	Mann-Whitney	0.1804
				WP85 PVP360	10	3.69	1.50	14		
			Shoots per explant (number)	WP83 PVP10	10	4.36	1.73	13	Mann-Whitney	0.3424
				WP85 PVP360	10	3.69	1.50	14		
S4-F16	14/05/2008	96	Weight gained (g)	WP31	10	0.43	0.64	12	Mann-Whitney	0.6439
				WP45	1	0.29	0.57	12		
			New shoots per initial shoot (number)	WP31	10	3.00	2.22	12	Mann-Whitney	0.2780
				WP45	1	2.42	2.23	12		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.17 Effect of activated charcoal (AC) on tissue cultures of *Gomortega keule*.

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	AC in medium (g/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S4-A3	21/05/2008	35	Shoots per explant (number)	WP81	0.0	2.33	1.00	9	Mann-Whitney	0.0265 *
				WP82	1.0	1.33	0.29	10		
S4-A3	23/05/2008	52	Shoots per explant (number)	WP65	0.0	3.13	2.07	15	Mann-Whitney	0.0213 *
				WP67	0.5	1.59	1.00	17		
			Shoots per explant (number)	WP66	0.0	2.87	2.39	15	Mann-Whitney	0.1762
				WP68	0.5	1.56	1.04	18		
			Explants with callus at the base of stem (number)	WP65 + WP66	0.0	0.50		30	Chi Square	0.0880
				WP67 + WP68	0.5	0.22		33		
S4-B6	23/05/2008	52	Shoots per explant (number)	WP65	0.0	1.78	1.20	9	Mann-Whitney	0.7390
				WP67	0.5	0.92	0.67	12		
			Shoots per explant (number)	WP66	0.0	2.00	1.12	9	Mann-Whitney	0.0459 *
				WP68	0.5	1.20	0.86	15		
S4-F16	14/05/2008	96	Weight gained (g)	WP31	0.0	0.43	0.64	12	Mann-Whitney	0.0006 **
				WP46	2.0	0.03	0.04	12		
			Shoots per explant (number)	WP31	0.0	3.00	2.22	12	Mann-Whitney	0.0041 **
				WP46	2.0	1.25	0.45	12		

Effect of activated charcoal (AC) on tissue cultures of *Gomortega keule* (continued).

Genotype	Evaluation date	Total time (d)	Variable measured (units)	Media	AC in medium (g/l)	Variable average value	SD	Number of replicates	Statistical test used	P value
S3-104	08/11/2008	123	Normal shoots per explant (number)	WP95	0.0	0.89	0.41	12	Kruskal-Wallis	0.5980
				WP96	1.0	0.86	0.41	12		
				WP97	2.0	0.97	0.26	12		
S3-109	08/11/2008	123	Normal shoots per explant (number)	WP95	0.0	0.27	0.36	11	Kruskal-Wallis	0.2620
				WP96	1.0	0.56	0.46	12		
				WP97	2.0	0.56	0.52	12		

Significant differences are indicated by asterisks (*, P< 0.05 and **, P<0.01).

9.18 Preparation of solutions for cryopreservation

Final volumes were adjusted to 100 ml using reverse-osmosis water (except for VSL solution), pH was adjusted to 5.8 and solutions were sterilized using a 0.2 µm Sartorius Minisart® syringe filter in a laminar flow cabinet.

Enriched-sucrose (0.3 M) WP medium

- general procedure for media preparation followed (Chapter 2), except for sucrose
- 102.688 g sucrose per litre of medium added
- no PGRs were added

Loading solution (0.4 M sucrose)

- 0.44 g MS medium salts
- 13.6918 g sucrose
- 18.4 g (final concentration 2.0 M) glycerol (Fisher)

Vitrification solution PVS2

- 0.44 g MS salts
- 13.6918 g sucrose (0.4 M)
- 30 g (3.26 M) glycerol
- 15 g (1.9 M) DMSO (Sigma)
- 15 g (2.42 M) ethylene glycol (Sigma)

Vitrification solution VSL (Suzuki *et al*. 2008)

- 5 g sucrose (0.15 M)
- 20 g glycerol (2.17 M)
- 10 g DMSO (1.28 M)
- 30 g ethylene glycol (4.83 M)
- 14.7 mg $CaCl_2$ dihydrate (10 mM) (Acros Organics, New Jersey, USA)
- final volume adjusted to 100 ml with MS liquid medium

Unloading solution (1.2 M)

- 0.44 g MS medium salts
- 41.075 g sucrose

Unloading solution, different molarities (only sucrose detailed)

- 6.846 g (0.2 M)
- 13.692 g (0.4 M)
- 27.384 g (0.8 M)

Alginate solution (for encapsulation)

- 3 % alginic acid sodium salt (Sigma)
- 30 g/l sucrose
- pH adjusted to 5.7
- the solution was autoclaved

Liquid MS medium with $CaCl_2$

- 11.1 g/l (0.1 M) $CaCl_2$ anhydrous (Sigma)
- 30 g/l sucrose
- pH adjusted to 5.7
- the solution was autoclaved

9.19 Preparation of solutions for chromosome observation

Methanol-acetic solution (methanol:glacial acetic acid, 4:1, v/v)

- 80 ml methanol (Fisher)
- 20 ml glacial acetic acid (Fisher)

TE buffer

- 1 ml 1M Tris pH 8.0 (prepared as detailed in Appendix 9.20)
- 0.2 ml 0.5 M EDTA pH 8.0 (prepared as detailed in Appendix 9.20)
- 99 ml reverse-osmosis water

Citrate buffer (0.01 M citric acid, 0.01M sodium citrate, pH 4.6)

- 226.2 mg citric acid diammonium salt (Sigma)
- 294.1 mg tri-sodium citrate (Fisher)
- pH adjusted to 4.6

Enzyme mixture (4% (w/v) Cellulase R10, 1% (w/v) Pectolyase Y23)

- 0.8 g Cellulase Onozuka R10 from *Trichoderma viride* (Duchefa)
- 0.2 g Pectolyase Y23 (Kyowa Chemical Products Co., Ltd., Osaka, Japan)
- final volume adjusted to 20 ml with citrate buffer
- aliquoted in centrifuge tubes (200 µl) and stored at -20ºC

9.20 Preparation of solutions for DNA extraction

Final volumes were adjusted using reverse-osmosis water.

Protocol of Lander *et al*. (2007) and modifications:

1.0 M Tris pH 8.0

- 12.11 g Tris base (Fisher) dissolved in 1.0 M HCl until pH 8.0
- final volume adjusted to 100 ml with water

0.5 M EDTA pH 8.0

- 18.612 g EDTA (BDH Laboratory Supplies, Poole, UK) dissolved in 75 ml water
- *ca.* 2 g of NaOH pellets (Acros Organics) dissolved
- pH adjusted to 8.0 with 1.0 M NaOH solution
- volume brought to 100 ml with water

5.0 M NaCl

- 29.22 g NaCl (Fisher) dissolved in 70 ml water
- final volume adjusted to 100 ml

CTAB solution

- 28 ml 5.0 M NaCl
- 4 ml 0.5 M EDTA pH 8.0
- 10 ml 1.0 M Tris pH 8.0
- 2 g CTAB (Sigma)
- 40 ml water

Chloroform:isoamylalcohol (24:1)

- 24 parts of chloroform (Fisher)
- 1 part of isoamylalcohol (Sigma)
- added and mixed under fume cabinet

Protocol of Rogers and Bendich (1985):

1x CTAB extraction buffer

- 0.5 g CTAB
- 5 ml 1.0 M Tris pH 8.0
- 2 ml 0.5 M EDTA pH 8.0
- 14 ml 5.0 M NaCl
- 0.5 g PVP 40,000 (Sigma)
- final volume was adjusted to 50 ml

10x CTAB

- 5 g CTAB
- 7 ml 5.0 M NaCl
- final volume brought to 50 ml in hot water bath

CTAB precipitation buffer

- 0.5 g CTAB
- 2.5 ml 1.0 M Tris pH 8.0
- 1 ml 0.5 M EDTA pH 8.0
- final volume adjusted to 50 ml

High salt TE buffer

- 0.5 ml 1.0 M Tris pH 8.0
- 0.1 ml 0.5 M EDTA pH 8.0
- 10 ml 5.0 M NaCl
- final volume brought to 50 ml

0.1x TE buffer

- 0.05 ml 1.0 M Tris pH 8.0
- 0.01 ml 0.5 M EDTA pH 8.0
- final volume adjusted to 50 ml

9.21 Preparation of solutions for gel electrophoresis

50x TAE buffer

- 242 g Tris base
- 57.1 ml glacial acetic acid (Fisher)
- 100 ml 0.5 M EDTA pH 8.0
- final volume brought to 1 litre

1x TAE buffer

- 20 ml 50x TAE buffer
- final volume adjusted to 1 litre

5x TBE buffer

- 54 g Tris base (Melford Laboratories Ltd., Ipswich, UK)
- 27.5 g Boric acid (Fisher)
- 20 ml 0.5 M EDTA pH 8.0
- final volume brought to 1 litre

0.5x TBE buffer

- 100 ml 50x TBE buffer
- final volume adjusted to 1 litre

9.22 Preparation of miscellaneous solutions

1x TE buffer for suspention of fluorescent labeled primers

- 1 ml 1M Tris base pH 8.0
- 0.2 ml 0.5 M EDTA pH 8.0
- 99 ml Water
- Filter sterilize and autoclave

6x Loading buffer

- 21 ml Water
- 9 ml Glycerol (Fisher)
- 75 mg Bromophenol blue sodium salt (Sigma)
- 75 mg Xylene cyanol FF (Sigma)

9.23 Matrix of alleles present in zygotic embryos

Tissue	Gk-1			Gk-5					Gk-6-1	
	224	226	230	307	311	315	317	319	98	106
Tree shoot	1	0	0	0	0	0	0	1	1	0
Embryo A	0	1	1	0	0	0	0	1	1	0
Embryo B	1	0	1	0	0	0	0	1	1	1
Embryo C	1	1	0	1	0	0	1	0	1	1
Embryo D	1	0	1	1	0	0	1	0	1	1
Embryo E	1	0	1	0	0	0	0	1	1	1
Embryo F	1	1	0	0	0	0	0	1	1	1
Embryo G	1	0	0	0	0	0	0	1	1	0
Embryo H	1	0	1	0	0	0	0	1	1	0
Embryo I	1	1	0	0	1	0	0	1	1	1
Embryo J	1	1	0	0	1	0	0	1	1	1
Embryo K	1	0	1	1	0	1	0	0	1	1

Tissue	Gk-6-2			Gk-30			Gk-31				Gk-35	
	104	114	116	182	186	188	215	222	224	226	226	228
Tree shoot	0	0	1	1	0	0	0	1	0	0	0	1
Embryo A	1	0	0	1	0	1	0	1	1	0	1	0
Embryo B	0	1	1	1	0	0	0	1	0	1	0	1
Embryo C	0	1	0	0	1	1	0	1	0	0	1	0
Embryo D	0	1	1	1	0	1	0	1	0	0	0	1
Embryo E	1	0	0	1	0	1	1	0	0	1	1	0
Embryo F	0	1	0	1	0	0	0	1	0	0	1	0
Embryo G	1	0	0	0	0	1	0	1	1	0	1	0
Embryo H	1	0	1	1	0	1	1	0	0	1	1	0
Embryo I	0	1	0	1	0	1	0	0	1	1	1	0
Embryo J	1	1	0	0	0	1	0	0	1	1	1	0
Embryo K	0	1	1	1	0	0	0	1	0	1	0	1

Tissue	Gk-39			Gk-44				CS2			CS8		
	132	188	201	155	156	157	159	104	117	119	210	212	218
Tree shoot	1	0	0	0	0	1	0	1	0	0	0	1	0
Embryo A	1	0	0	0	1	0	1	0	1	1	1	1	0
Embryo B	1	0	0	0	1	0	1	1	0	1	0	1	0
Embryo C	1	1	0	0	1	0	0	1	1	0	1	1	0
Embryo D	1	0	0	0	1	0	0	0	1	0	0	1	0
Embryo E	1	0	0	1	0	0	0	1	1	0	1	1	0
Embryo F	1	0	1	1	0	0	0	0	1	1	0	1	1
Embryo G	1	0	0	0	1	0	0	0	1	0	1	1	0
Embryo H	1	0	0	0	1	0	0	0	1	1	0	1	0
Embryo I	1	0	1	0	0	1	0	0	1	1	1	1	0
Embryo J	1	0	0	0	0	1	0	0	1	0	0	1	0
Embryo K	1	0	0	0	1	0	1	1	1	0	0	1	0

9.24 Linkage disequilibrium test for each pair of loci per population

Population BA

Loci	Gk-6-1	Gk-6-2	Gk-30	Gk-31	Gk-35	Gk-39	CS2	CS8
Gk-1	*	*	*	*	-	-	-	-
Gk-6-1		*	-	-	-	*	-	-
Gk-6-2			-	-	-	-	*	-
Gk-30				*	-	*	*	-
Gk-31					*	-	*	-
Gk-35						-	*	*
Gk-39							-	-
CS-2								*

Population FTC

Loci	Gk-6-1	Gk-6-2	Gk-30	Gk-31	Gk-35	Gk-39	CS2	CS8
Gk-1	-	-	-	-	-	-	-	-
Gk-6-1		*	-	-	-	*	*	-
Gk-6-2			*	-	-	*	*	-
Gk-30				-	-	*	*	*
Gk-31					-	-	-	-
Gk-35						-	-	-
Gk-39							-	-
CS-2								-

Population North

Loci	Gk-6-1	Gk-6-2	Gk-30	Gk-31	Gk-35	Gk-39	CS2	CS8
Gk-1	*	-	-	-	-	-	-	-
Gk-6-1		*	-	-	-	-	-	-
Gk-6-2			*	-	*	-	-	*
Gk-30				-	-	*	-	*
Gk-31					*	-	-	-
Gk-35						-	-	*
Gk-39							-	-
CS-2								-