Vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), a Key Pest in South African vineyards. A Review

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Vine mealybug, *Planococcus ficus* (Signoret), is a key pest in vineyards in the Western Cape and North-West Provinces of South Africa and more recently in the USA. This pest was first reported in the Western Cape Province in 1943. The taxonomy and identification of this species are made difficult by complex slide-mounting techniques and the lack of qualitative characteristics. Vine mealybug is polyphagous with a wide range of host plants. *P. ficus* causes direct crop loss due to desiccation of bunches in the case of wine grapes and unsightly honeydew excretion on bunches in the case of table grapes. High infestations of *P. ficus* can cause early leaf loss and resultant weakening of vines. Vine mealybug also vectors the vine leafroll virus. This pest is currently controlled using chemical, biological and cultural control techniques in an integrated pest-management system. This system relies on the use of pheromone and physical monitoring techniques, which provide information on infestation levels.

INTRODUCTION AND HISTORY OF THE PEST IN SOUTH AFRICA

Planococcus ficus (Signoret) was initially identified in the Western Cape Province as *Planococcus citri* (Risso) by Joubert (1943), Kriegler (1954) and Whitehead (1957) after introduction to the area, probably with plant material. De Lotto (1975) subsequently identified it as *Planococcus ficus*. The most recent samples of the insect collected during 1999/2000 were identified as *Planococcus ficus* (Walton 2003) by I.M. Millar, Plant Protection Research Institute in Pretoria.

P. ficus was first recorded as a pest in the Western Cape Province during 1930 (Joubert 1943). By 1935 *P. ficus* had spread to the Hex River Valley and subsequently to all other major grape-producing areas (Joubert 1943) in this Province. Kriegler (1954) regarded it as one of the most important pests of the grape industry in South Africa. Other pseudococcid species recorded from vines in the Western Cape Province included *Pseudococcus longispinus* (Targioni) and *Ferrisia malvastra* (McDaniel), which were also identified by I.M. Millar (Walton 2003), Plant Protection Research Institute in Pretoria. These have, however, not yet attained pest status on grapes locally.

TAXONOMY

Current Status

The most recent classification was done by Ben-Dov (1994) who placed *P. ficus* in the Order Hemiptera, Suborder Homoptera, Superfamily Coccoidea and Family Pseudococcidae. The species was well described by De Lotto (1975), Cox (1981, 1989), and Williams & Granara de Willink (1992). Keys for the adult female of this species were given in Williams & Moghaddam (1999) (Iran), Williams & Granara de Willink (1992) (Central and South America), Cox (1989) (World), Cox & Ben-Dov (1986) (Mediterranean basin), and Cox & Wetton (1988) (West Indies). *P. ficus* was initially described as *Coccus vitis* by Nedzilskii (1869) (Cox & Ben-Dov, 1986). Lichtenstein (1870) subsequently placed this species in the genus *Dactylopius* (Cox 1989). Signoret (1875) described it as *Planococcus ficus*. Thereafter various synonyms were used, many of which were the result of misidentification (Ben-Dov, 1994) (Table 1).

Vernacular names

Vernacular names given by Balachowsky & Mesnil (1935) include 'cocciniglia farinosa della vite', 'cochonilha algodeo da vinha', 'cotonet de la vid', 'grapevine mealybug' and 'la cochenille farineuse de la vigne'. Berlinger (1977) described *P. ficus* as the 'Mediterranean vine mealybug', Bodenheimer (1924) as 'subterranean vine mealy bug' and De Lotto (1975) as 'vine mealybug'.

BIOLOGY

Morphometrics

Criteria for age distinction of the different developmental stages of *P. ficus* were described by Kriegler (1954). This information was used in studies on the developmental biology of this pest (Walton, 2003). Kriegler (1954) made use of a combination of colour, size, and other characteristics to distinguish between the different stages. Certain criteria were selected and are presented in Table 2.

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TABLE 1
Synonyms used for *Planococcus ficus* (Ben-Dov 1994).

Synonym	Author	Comment
Coccus vitis	Nedzilskii (1869), Lindinger (1912), Borchsenius (1949)	Incorrect due to misidentification (Cox & Ben-Dov, 1986). True identity unknown.
Dactylopius vitis	Lichtenstein (1870), Signoret (1895)	Misidentification (Cox, 1989)
Dactylopius ficus	Signoret (1875), Borchsenius (1949)	Type material lost (Ben-Dov & Matile-Ferrero, 1995).
Dactylopius subterraneus	Hempel (1901)	On roots of cultivated grapes
Pseudococcus ficus	Fernald (1903)	Change of combination
Pseudococcus vitis	Fernald (1903), Leonardi (1920), Bodenheimer (1924)	
Pseudococcus citriodes	Ferris (1922)	New name
Pseudococcus citri	Balachowsky & Mesnil (1935)	Misidentification
Dactylopius ficus	Borchsenius (1949)	Synonymous with *Pseudococcus citri* (Risso)
Planococcus citroides	Ferris (1950)	Change of combination
Planococcus vitis	Ezzat & McConnell (1956), Matile-Ferrero (1984)	
Planococcus ficus	Ezzat & McConnell (1956)	Change of combination
Pseudococcus praetermissus	Ezzat (1962)	Synonym

In a recent survey (Walton, 2003), *P. ficus* was found to be the dominant mealybug species in vineyards in the Western Cape Province of South Africa. Adult female mealybugs were approximately 4 mm in length, slightly more than 2 mm wide and about 1.5 mm thick. The adult female and immature stages were ovate, humpbacked, light slate- to flesh-coloured and covered by a fine, white powdery wax secretion, which was more evident on the later stages. The body of the adult female was clearly segmented and had a fringe of short, finger-like wax filaments around its edge (Kriegler, 1954) (Fig. 1). After mating, egg sacs covered with waxy threads started to appear.

This species was easily distinguished from *Ps. longispinus*, which was about 3 mm long, 1 mm wide, ovate, and yellowish grey in colour. Adult females and younger stages of this species had exceptionally long posterior filaments and no egg sacs, as this species was ovoviviparous (El-Minshawy, *et al.* 1974). A single adult female *Ferrisia malvastra* (McDaniel), 7 mm long and 4 mm wide with a light orange colour was recorded for the first time from a vineyard in Stellenbosch (Walton, 2003) and clearly differed in morphology from *P. ficus*.

P. ficus is closely related to *P. citri*. Separation of these two species is based on minor differences in the number and arrangement of glandular ducts of the dermis. *P. ficus* has fewer groups of and smaller ducts than *P. citri* (De Lotto, 1975). However, *P. citri* has not yet been found on vines in South Africa.

Life cycle

Kriegler (1954) studied the lifecycle of *P. ficus* in detail. Developmental stages studied were eggs and first, second and third nymphal instars. It was found that the male characteristics appeared after the third nymphal instar and, during subsequent development, differentiation between the sexes occurred. In the case of the male, the prepupa was followed by the pupa from which the winged male emerged (Fig. 1). Males were characterised by having long filamentous anal setae and no mouthparts (Kriegler 1954). The adult female started releasing pheromones at sexual maturity, attracting adult males for copulation (Hinkens *et al.*, 2001). Subsequent to copulation there was a pre-oviposition period, after which the female laid eggs in an egg sac made up of

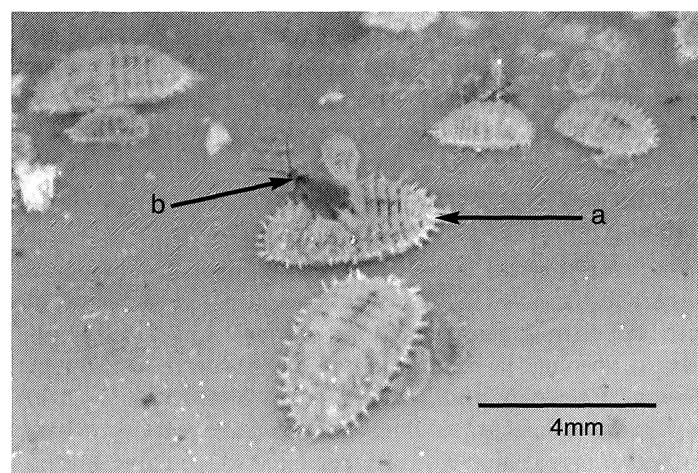

FIGURE 1
Adult female (indicated by arrow a) and male (indicated by arrow b) *Planococcus ficus*.

TABLE 2

Morphometric characters for distinguishing life stages of *Planococcus ficus* (Kriegler, 1954) in developmental biology studies.

Stage	Average length (mm)	Average width (mm)	Characteristics/Colour
Egg	0.41	0.21	Light straw
First nymphal instar	0.46	0.22	Light to dark yellow, six antennal segments
Second nymphal instar	0.68	0.35	Yellowish brown
Third nymphal instar	1.13	0.66	Seven antennal segments
Male prepupa	0.95		One pair of lateral ocelli. Visible wingbuds
Male pupa	1.05		Three pairs of lateral ocelli. Wingbuds reaching to third abdominal segment
Adult male	1.05		Wings fully developed
Adult female	1.69	0.99	Wingless, eight antennal segments

filamentous waxy hairs. Kriegler (1954) recorded an average of 362 eggs per female. Life-table studies were done at constant temperatures by Walton (2003) whereby the lower and upper threshold temperatures for development of *P. ficus* were estimated at 16.59 and 35.61°C, respectively. These results were similar to those of Duso *et al.* (1985), who indicated that the optimum temperatures ranged from 23°C to 27°C.

Hosts

P. ficus is a polyphagous insect and, apart from the economic damage it can cause to *Vitis vinifera* Linn., it has been found on various other host plants (Table 3).

None of the above host plants were found in close proximity to the vineyards sampled in the study by Walton (2003). A variety of weeds were, however, sampled for mealybugs in vineyards during the current study, but no *P. ficus* were found on any of them (Walton 2003).

GEOGRAPHICAL DISTRIBUTION AND ECONOMIC IMPORTANCE

P. ficus has been found in most grape-production areas throughout the world (Table 4). It is of particular economic importance on grapevines in the Mediterranean region, South Africa, Pakistan and Argentina (Ben-Dov, 1994).

Engelbrecht & Kasdorf (1984) and Cabaleiro & Segura (1997) found that *P. ficus* transmitted the grapevine leafroll associated virus 3 (GLRa V-3). Initially, the mealybug specimens studied by Cabaleiro & Segura (1997) were identified as *Planococcus citri* (Risso), but later identified by Ben-Dov as *P. ficus* (Signoret) (Yair Ben-Dov, unpublished data, July 1998). Transmission of GLRa V-3 by *P. ficus* and positive identification of GLRa V-3 were further confirmed using PCR methods by Acheche *et al.* (1999).

The transfer of the leafroll virus caused inefficient photosynthesis, which resulted in reduced fruit production, inability to produce sufficient sugar, higher than normal acidity levels and delayed ripening. In addition, infested vines were less drought resistant (Cabaleiro *et al.*, 1999; Manini, 2000). Manini (2000) showed that uninfected seedlings had increased vegetative vigour and higher propagation potential than infected seedlings. In addition, *P. ficus* has been found to be a virus vector of corky-bark disease (Engelbrecht & Kasdorf, 1985; Tanne *et al.*, 1989) and Shiraz disease (Engelbrecht & Kasdorf, 1984) in vines.

Apart from being a vector of GLRa V-3, high infestations of *P. ficus* in table grape bunches result in direct crop loss and progressive weakening of vines through early leaf loss (Kriegler, 1954; Whitehead, 1957; Berlinger, 1977; Charles, 1982; Walton, 2003).

Seasonal population dynamics and phenology

In South Africa Kriegler (1954) recorded six generations per year, while Walton (2003) found between five and six generations. In Italy Duso (1990) recorded only three generations per year. These differences could be attributed to temperature differences between the two countries.

Upward movement on the trunk began from spring or early summer (October in South Africa, March/April in Israel and Italy) (Kriegler, 1954; Berlinger, 1977; Duso, 1990; Walton, 2003). Populations started to develop on new growth and the population peak was recorded between the end of January and the beginning of February, after which numbers declined (Kriegler, 1954; Whitehead, 1957; Walton, 2003). Mealybugs found in the vine canopy after harvest formed the nuclei of winter colonies (Whitehead, 1957). Similar observations were made in Israel and Italy (Berlinger, 1977; Duso, 1990). Berlinger (1977) noted that winter population levels were low in Israel and consisted mainly of non-ovipositing adult females. Walton (2003) found populations of this pest on roots of vines.

Kriegler (1954) studied the influence of temperature on the development of *P. ficus* under fluctuating temperatures on potatoes in an outdoor greenhouse. Duso *et al.* (1985) and Berlinger (1977) studied the development of *P. ficus* in the field. Berlinger (1977) found that cool early summer temperatures delayed upward migration, which delayed the population peak.

MONITORING SYSTEMS FOR VINE MEALYBUG

The low tolerance for *P. ficus* and the importance of timely insecticide applications necessitated the use of a species-specific monitoring programme for rapidly determining the pest population density. Two monitoring systems are currently in use: labour-intensive (Geiger & Daane, 2001) physical sampling of vines infested with *P. ficus* (Walton 2003) and pheromone monitoring (Millar *et al.*, 2002; Walton *et al.*, 2003).

Physical sampling can be used by producers in South Africa to provide an estimate of *P. ficus* population levels in commercial vineyards with known levels of error, enabling producers to

TABLE 3

Recorded findings of *P. ficus* on host plants other than *V. vinifera.*

Family	Genus/Species	Reference
Anacardiaceae	*Mangifera indica* Blume	Ezzat & McConnel (1956), Cox (1989), Ben-Dov (1994)
Apocynaceae	*Nerium oleander* Linn.	Ezzat & McConnel (1956)
Asteraceae	*Dahlia* spp.	Ezzat & McConnel (1956)
Juglandaceae	*Juglans* spp.	Ezzat & McConnel (1956)
Lauraceae	*Persea americana* Mill.	Cox (1989), Ben-Dov (1994)
Labiaceae	*Dichrostachys glomerata* Linn.	Cox (1989), Ben-Dov (1994)
	Prosopis farcata Linn.	Cox (1989), Ben-Dov (1994)
	Tephrosia purpurea Pers.	Cox (1989), Ben-Dov (1994)
Moraceae	*Ficus benjamina* Linn.	Williams & Granara de Willink (1992), Ben-Dov (1994)
Palmae	*Phoenix dactylifera* Linn.	Cox (1989), Ben-Dov (1994)
Platanaceae	*Platanus orientalis* Linn.	Martin-Mateo (1985), Williams & Moghaddam (1999)
Poaceae	*Bambusa* spp.	Ezzat & McConnel (1956)
Rhamnaceae	*Zizyphus spina-christi* Georgi	Cox (1989), Ben-Dov (1994)
Rosaceae	*Cydonia oblonga* Mill.	Granara de Willink *et al.* (1997)
Rosaceae	*Malus domestica* Baumg.	Granara de Willink *et al.* (1997)
	Malus pumila Mill.	Cox (1989), Ben-Dov (1994)
Salicaceae	*Salix spp.*	Cox (1989), Ben-Dov (1994)
Sterculiaceae	*Theobroma cacao* Linn.	Ezzat & McConnel (1956)
Styracaceae	*Styrax officinalis* Walt.	Cox (1989), Ben-Dov (1994)

decide on the necessity for and correct timing of intervention (Walton, 2003). Physical monitoring methods are most effective later in the summer, when mealybugs are in exposed locations (e.g. new canes and leaves) and when the population densities are relatively high (Geiger *et al.*, 2001; Walton 2003). In practice, this period occurs only after crop damage has taken place.

Pheromone-based monitoring programmes are less time consuming, simpler and more sensitive than physical inspections (Millar *et al.*, 2002; Walton *et al.*, 2003). Pheromone-baited lures were found to be attractive to male *P. ficus* for up to 12 weeks, with an effective range of 50 m (Hinkens *et al.,* 2001). The number of *P. ficus* males caught in pheromone-baited traps was positively correlated to female mealybug infestation levels, determined using physical sampling methods (Walton *et al.*, 2003).

CONTROL STRATEGIES

Chemical control

Chemical control of *P. ficus* in South Africa is currently based on either two treatments of chlorpyrifos two weeks apart, or prothiophos just before bud burst. These treatments are applied during the dormant period. An additional supplementary treatment of a chemical with a short residual period, such as dichlorvos or methidathion, is sometimes applied prior to harvest from January to April (Nel *et al.*, 1999). However, *P. ficus* colonies are protected by wax threads and are not easily controlled by these routine sprays. Populations usually occur under bark and in crevices on the main stem as well as on roots, making it difficult to target this pest with insecticides (Berlinger, 1977). Kriegler (1954) and Whitehead (1957) recommended the application of spot treatments with chemicals when high mealybug infestations occur. However, they emphasised the integrated use of chemical and biological control. The systemic pesticide Imidacloprid SC (350g/L) (http://www.ipw.co.za) was recently registered for use on vine mealybug in South Africa. This chemical may be a useful alternative to the chemicals mentioned above. However, there are indications that pesticide resistance to this compound can develop (Prabhaker *et al.*, 1997; Zhao *et al.*, 2000). Therefore, resistance-management measures should be employed to delay or prevent the development of resistance.

Biological control

Many natural enemies associated with *P. ficus* have been reported, some of which were hyperparasitoids (Table 5).

From the list it is clear that *P. ficus* populations are attacked by a range of natural enemies, many of which commonly occur in the Western Cape Province (Whitehead, 1957; Urban, 1985; Walton, 2003). The most common natural enemies in this area include, in descending order of abundance,

- Parasitoids: *Anagyrus* spp., *Coccidoxenoides perminutus*, *Leptomastix dactylopii.*
- Predatory beetles: *Nephus bineavatus*, *N. angustus* and *N. quadrivittatus*.

TABLE 4

Geographical areas where *Planococcus ficus* has been recorded on vines (Ben-Dov 1994).

Geographical area	Reference
Afrotropical: South Africa	Ezzat & McConnel (1956), De Lotto (1975), Cox (1989), Ben-Dov (1994)
Mauritius	Ezzat & McConnel (1956)
Nearctic: United States of America	Ezzat & McConnel (1956)
Neotropical: Argentina	Hempel (1901), Ezzat & McConnel (1956), , Granara de Willink (1991), Williams & Granara de Willink (1992), Ben-Dov (1994), Trjapitzyn & Trjapitzyn (1999)
Brazil	Williams & Granara de Willink (1992), Ben-Dov (1994)
Chile	Ezzat & McConnel (1956)
Dominican Republic	Ezzat & McConnel (1956)
Trinidad and Tobago	Ezzat & McConnel (1956)
Uruguay	Granara de Willink *et al.* (1997)
Oriental: India	Varshney (1992), Ben-Dov (1994)
Pakistan	Cox (1989), Ben-Dov (1994)
Palearctic: Afghanistan	Kozár, Fowjhan & Zarrabi (1996)
Azerbaijan	Rzaeva (1985), Ben-Dov (1994)
Azores	Ezzat & McConnel (1956)
Canary Islands	Carnero Hernandez & Pérez Guera (1986), Pérez Guerra & Carnero Hernandez (1987), Ben-Dov (1994)
Palearctic: Crete	Argyriou (1983), Cox (1989), Ben-Dov (1994)
Cyprus	Cox (1989), Ben-Dov (1994)
Egypt	Ezzat & McConnel (1956), Ezzat & Nada (1987), Cox (1989), Ben-Dov (1994)
France	Signoret (1875), Ben-Dov (1994)
Greece	Ezzat & McConnel (1956)
Hyeres Islands	Foldi (2000)
Iran	Cox (1989), Ben-Dov (1994), Kozar, Fowjhan & Zarrabi (1996), Williams & Moghaddam (1999)
Iraq	Cox (1989), Ben-Dov (1994)
Israel	Bodenheimer (1924), Avidov (1961), Avidov & Harpaz 1969), Cox & Ben-Dov (1986), Ben-Dov (1994)
Italy	Leonardi (1920), Tranfaglia (1976), Marotta (1987), Rosciglione & Castellano (1985), Duso (1990), Ben-Dov (1994)
Lebanon	Cox (1989), Ben-Dov (1994)
Libya	Ferris (1922), Ben-Dov (1994)
Portugal	Ezzat & McConnel (1956)
Sardinia	Melis (1930), Ben-Dov (1994), Longo *et al.* (1995), Pellizzari-Scaltriti & Fontana (1996)
Saudi Arabia	Beccari (1971), Matile-Ferrero (1984), Ben-Dov (1994)
Sicily	Longo *et al.* (1995), Russo & Mazzeo (1997)
Spain	Gómez-Menor Ortega (1937), Ezzat & McConnel (1956), Martin-Malteo (1985), Ben-Dov (1994)
Syria	Ezzat & McConnel (1956)
Tunisia	Cox (1989), Ben-Dov (1994)
Turkmenistan	Achangelskaya (1930), Ben-Dov (1994)

Berlinger (1977) also found that the parasitoids and predators mentioned above were dominant in Israel. Whitehead (1957) believed that predatory beetles played a major part in biological control and that the parasitoids were of lesser importance. Predatory beetle population levels peaked early in the season (from September to November) and declined after this (Walton, 2003; Whitehead, 1957). However, mealybug population levels did not decrease while the predators were present (Berlinger,

TABLE 5

Natural enemies associated with *P. ficus*.

Order and Family	Species	Reference	Comment
Diptera: Chamameyidae	*Leucopis* sp.	Rzaeva (1985)	
Hymenoptera: Encyrtidae	*Pachyneuron concolor* Forster	Rzaeva (1985)	Possible hyperparasitoid
	Allotropa mecrida Walker	Rzaeva (1985)	
	Anagyrus pseudococci (Girault)	Rzaeva (1985), Urban (1985), Trjapitzyn & Trjapitzyn (1999)	
	Chartocerus subaeneus Forster	Rzaeva (1985)	Possible hyperparasitoid
	Clausenia josefi Rosen	Rosen (1965), Berlinger (1977), Trjapitzyn (1989)	
	Coccidoxenoides perminutus (Timberlake)	Berlinger (1977), Urban (1985), Trjapitzyn (1989)	Synonym: *Pauridia peregrina*
Hymenoptera: Encyrtidae	*Leptomastix flavus* Mercet	Berlinger (1977)	
	Leptomastidea abnormis (Girault)	Berlinger (1977), Urban (1985), Trjapitzyn (1989), Trjapitzyn & Trjapitzyn (1999)	
	Prochiloneurus bolivari (Mercet)	Trjapitzyn (1989)	Possible hyperparasitoid
	Prochiloneurus pulchellus (Silvestri)	Trjapitzyn (1989)	Possible hyperparasitoid
	Chrysoplatycerus splendens (Howard)	Walton (2003)	
Neuroptera: Chrysopidae	*Chrysoperla carnea* (Stephens)	Rzaeva (1985)	
Coleoptera: Coccinellidae	*Nephus reunioni* Fürsch	Rzaeva (1985)	
	Cryptolaemus montrouzieri Mulsant	Orlinskii *et al.* (1989)	
	Hyperaspis felixi Mulsant	Whitehead (1957), Urban (1985)	
	Nephus angustus Casey	Whitehead (1957), Urban (1985)	
	Nephus binaevatus Mulsant	Whitehead (1957), Urban (1985)	
Coleoptera: Coccinellidae	*Nephus quadrivittatus* Mulsant	Whitehead (1957), Urban (1985)	
	Rhizobiellus sp.	Whitehead (1957)	
	Cydonia lunata F.	Whitehead (1957)	
	Scymnus nubilis Mulsant	Walton (2003)	

1977; Urban, 1985; Walton, 2003) both in the Western Cape and in Israel. The lack of density-dependence documented between vine mealybugs and predatory beetles led Walton (2003) to assume that this group of beneficials were of lesser importance. Parasitoid numbers reached a peak later in the season (from November), which resulted in the destruction of most of the mealybug colonies (Berlinger, 1977; Urban, 1985; Walton, 2003) towards the end of the season (February to March). This suggested that the parasitoid complex played a major role in reducing *P. ficus* numbers.

Biological control was severely hampered by the presence of a variety of ant species (Kriegler, 1954; Whitehead, 1957; Addison & Samways, 2000) in vineyards in the Western Cape Province. This was also reported in Israel (Berlinger, 1977). Ant control has been achieved using chemical stem-barrier treatments (Addison, 2002). Walton (2003) did a two-year field study of mass releases

of *C. perminutus*, a parasitoid of the first, second and third instars of *P. ficus*. This method of control was at least as effective as the currently used chemical control programme.

Cultural control

Bugg & Waddington (1994), Whitehead (1957) and Urban (1985) suggested that the preservation of surrounding vegetation was important for optimising conditions for natural enemies. Cover crops were effective only if they attracted Coccinellidae and Neuroptera (Bugg & Waddington, 1994). These authors also noted that common vetch (*Vicia sativa*) had stipular extra-floral nectaries that attracted parasitic wasps. Work done on the effects of cover crops on natural enemy populations of mealybugs by P. Addison (Personal communication) and Costello & Daane (2003) indicated that cover cropping had no significant effect on their occurrence in vineyards.

Providing pollen, nectar, suitable habitats, sprays of sucrose or a yeast product plus sucrose led to an increase in local populations of predatory coccinellids, chrysopids, and hemerobiids. These food sources increased the longevity not only of predators, but also of adult encyrtid wasps and enhanced biocontrol of mealybugs in the field (Neuenschwander & Hagen, 1980; Urban, 1985).

Kriegler (1954) and Flaherty *et al.* (1982) found that leaf removal and correct summer pruning reduced the number of leaves that predators and parasitoids had to cover in search of prey, thereby increasing their effectiveness. This also reduced mealybug populations by removing them with the surplus stems and leaves, and contributed to better aeration of vines. Road dust and inert carriers of fungicides should be kept to a minimum as these adversely affected natural enemies (Searle, 1965). Mealybugs overwintering on old wood and under loose bark readily infested bunches that touched the woody parts of the vine. However, bunches that hung free from old wood were less susceptible to cosmetic damage. Therefore, bunches touching the old wood should be thinned so as to avoid contact (Kriegler, 1954; Flaherty *et al.*, 1982). The use of chemical and sticky trunk barriers to keep ants from the vine canopy could further aid in biological control of *P. ficus* (Whitehead, 1957; Addison, 2002).

The spread of *P. ficus* pest populations can further be limited by co-ordinating on-farm movement of implements and labourers.

Integrated control

Whitehead (1957), Berlinger (1977) and Urban (1985) believed that an integrated approach should be followed, which would enhance biological control. In addition, ant exclusion by trunk barriers was considered an important element of the integrated system (Whitehead, 1957). If biological control was not adequate, limited chemical intervention using spot treatments of short residual pesticides should be considered.

Information on the development rate of *P. ficus* (Walton, 2003) was used to estimate the number of degree-days required by *P. ficus* to complete its development and to estimate the rate of development of the *P. ficus* population through the season (Walton, 2003). This information was used as input for a *P. ficus* pest-management model. Data from monitoring *P. ficus* and ant activity were used as components to construct a decision chart. This chart can be used by producers to optimise the control of *P. ficus* populations using either chemical control or mass releases of *C. perminutus.*

Presently, integrated production of wine (IPW) is encouraged by the wine industry in South Africa (Tromp & Marais, 2000). This system includes sound integrated pest-management strategies for suppressing pests such as *P. ficus*. Strategies include monitoring pest activity, pest-control practices such as trunk barriers, optimised use of biological control and limited use of chemicals during the growing season. In addition, an AgChem Environmental Work Group codes all registered pesticides for acceptability in integrated production systems for use against insect pests, including those for *P. ficus* control. This coding system is based on the environmental impact of products (Walton & Pringle, 1999; Tromp & Marais, 2000; Walton & Pringle, 2001). Producers are encouraged to implement these guidelines (www.ipw.co.za) and random audits are conducted to determine compliance.

CONCLUSIONS

The taxonomic status of *P. ficus* has been uncertain due to the difficulty of the slide-mounting techniques used for preparing specimens for identification and the lack of qualitative physical differences to other closely related species (De Lotto, 1975; Ben-Dov, 1994). A recent survey of mealybugs in the Western Cape Province indicated that *P. ficus* is dominant locally (Walton, 2003). This has important implications for the grape-growing industry in South Africa, because vine mealybug is an important vector of the vine leafroll virus. The spread of this virus can only be controlled by limiting the development of *P. ficus* infestations in vineyards.

The majority of producers currently control this pest using commercially registered pesticides. The recent registration of the chloro-nicotinyl compound, imidacloprid, and possible registration of similar chemical compounds in the future may aid in alleviating the limited range of available chemical compounds for vine mealybug control. The optimal application rate, timing, cost effectiveness and efficacy of these compounds, however, need to be determined.

The current global trend of antagonism towards pesticide use, the evidence of pesticide resistance and the difficulty of controlling this pest with conventional pesticides, however, serve as an incentive for using alternative pest-control strategies. An integrated control programme is seen as the only sustainable alternative to the currently used chemical control regime. Tools available for integrated control include physical and pheromone-baited monitoring, temperature-driven models, biological control through mass releases of natural enemies and optimally timed chemical sprays. The isolation and commercial synthesis of the vine mealybug pheromone have provided an opportunity to investigate mating disruption as a further alternative pest-management strategy for the control of *P. ficus*.

The lower and upper developmental temperatures of *P. ficus* and *C. perminutus*, an important parasitic wasp, have been determined (Walton, 2003). These parameters were used to estimate the number of degree days required for both insects to complete their entire lifecycles. This information can be used in temeperature-driven models for optimising the timing of control measures.

Pesticide failures have necessitated the development of alternative pest-management measures such as mass releases of natural enemies for *P. ficus* control in South Africa (Walton, 2003). A

w of the published information on mass rearing parasitoids ɔeen produced by Etzel & Legner (1999), but no literature ɪvailable on the mass rearing of *C. perminutus* on *P. ficus*. To ɪote biological control as an alternative to chemical control, *ɾrminutus* was produced and released on *P. ficus* pest popula- and methods for mass release and the effectiveness of *ɜrminutus* for controlling *P. ficus* populations were investi- l (Walton, 2003).

time control actions such as mass releases or chemical con- ıf *P. ficus* pest populations correctly, accurate information on infestation levels was needed and a system for monitoring *P.* population levels with known levels of error was developed ; pheromone and physical monitoring (Walton, 2003). With ɔove information, action thresholds could be determined and as a basis for *P. ficus* management.

ormation gathered on the above aspects was combined to ruct a decision model for integrated *P. ficus* management ːon, 2003). This decision model should be verified in the Future work will include the use of *P. ficus* pheromones for ıg disruption.

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active transport via a malate permease, and an effective L-malic acid-converting enzyme, such as the malic enzyme. The malate permease gene (*mae1*) and the malic enzyme gene (*mae2*) of *S. pombe* were therefore cloned (Viljoen *et al.*, 1994; Grobler *et al.*, 1995) and co-expressed in multi-copy and single copy under the *S. cerevisiae* constitutive 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences in a laboratory strain of *S. cerevisiae* (Volschenk *et al.*, 2001). A strong malo-ethanolic phenotype was introduced in *S. cerevisiae,* where L-malic acid was rapidly and efficiently degraded in synthetic and Chardonnay grape must with the concurrent production of higher ethanol levels (Volschenk *et al.*, 2001). Functional expression of the malo-ethanolic pathway genes of *S. pombe* in a laboratory strain of *S. cerevisiae* paved the way for the genetic modification of industrial wine yeast strains of *Saccharomyces* for commercial winemaking applications.

Stable integration of the malo-ethanolic pathway genes into the genome of industrial wine yeast strains is a prerequisite for becoming an inherited component of the yeast genome. Genetic engineering of wine yeast strains of *Saccharomyces* is, however, complicated by the homothallic, multiple ploidy and prototrophic nature of industrial strains of *Saccharomyces* (Pretorius, 2000). Transformation and stable integration of heterologous genes into industrial strains of *Saccharomyces* require the use of dominant selectable markers, antibiotic or toxic compound resistance markers. However, integration of these markers into the yeast genome is not acceptable for commercial application, mainly due to the absence of long-term risk assessment and to consumer disapproval.

The integration of the malo-ethanolic expression cassettes in industrial wine yeast strains previously reported by Volschenk *et al.* (2001) was based on resistance to the herbicide sulphometuron methyl (SMM) via the *SMR1-410* gene. In this study we report a novel integration strategy for the *S. pombe mae1* and *mae2* expression cassettes without the incorporation of any non-yeast derived DNA sequences. Integration and expression of the malo-ethanolic genes in *S. cerevisiae* S92 resulted in rapid and complete degradation of L-malic acid and increased ethanol production during the early stages of alcoholic fermentation. Furthermore, this had no adverse effect on the yeast's fermentative ability, and sensory evaluation and chemical analysis of Chardonnay wine indicated an improvement in wine flavour perception compared to the control wines.

MATERIALS AND METHODS

Strains and maintenance

The microbial strains and plasmids used in this study are listed in Table 1. Cells of *Escherichia coli* JM109 were transformed by electroporation and selected on LB agar medium supplemented with 200 mg/L ampicillin (Ausubel *et al.*, 1995). *S. cerevisiae* S92 (a galactose positive strain of *S. cerevisiae* previously referred to as *Saccharomyces bayanus*) was maintained on YPD agar, while transformants were plated onto YEG media containing 0.5% yeast extract, 2% glucose, 3% Pastagar B (Difco Laboratories, Detroit, MI) and 250 µg/mL phleomycin.

Plasmid construction

Standard recombinant DNA techniques were employed essentially as described by Ausubel *et al.* (1995). Restriction enzymes, modification enzymes and DNA purification kits were used as prescribed by the manufacturer (Roche Diagnostics, Germany). All polymerase chain reactions (PCR) were executed with Takara Ex *Taq* (Takara Bio Inc, Japan). All subcloning and DNA manipulations were performed in the 2µm-based plasmid YEp352 (Hill *et al.*, 1986). Prior to any subcloning, the *Kpn*I restriction site located in the multiple cloning region of YEp352 was eliminated

TABLE 1

Strains and plasmids used for the integration of the *S. pombe* malo-ethanolic genes into a commercial strain of *Saccharomyces cerevisiae.*

Strains	Description	Reference
***E. coli* JM109**	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 $[r_k-, m_k+]$, *relA*1, *supE*44, λ^-, Δ(*lac-proAB*), [F', *traD*36, $proA^+B^+$, $lacI^qZ\Delta M15$]	Yanisch-Perron, 1985
***S. cerevisiae* S92**	Commercial wine yeast	Lesaffre
Plasmids	**Description**	**Reference**
YEp352	Yeast/*E. coli* shuttle vector with a *URA3* marker	Hill *et al.*, 1986
pUT332	Yeast episomal plasmid containing the *Tn5ble* gene for selection of phleomycin resistance	Gatignol *et al.*, 1990; Wenzel *et al.*, 1992
pHV3	pHVX2 containing the *mae1* ORF subcloned between the *PGK1* promoter and terminator sequences.	Volschenk *et al.*, 1997[a, b]
pHV7	YEplac195 (*URA3* marker gene) containing the *mae2* ORF subcloned between the *PGK1* promoter and terminator sequences.	Volschenk *et al.*, 2001
pHV9	YEp352 without the *Kpn*I restriction site	This study
pHVJH1	pHV9 containing the mutated *URA3* gene	This study
pHV11	pHVJH1 containing the $PGK1_p$-*mae1*-$PGK1_t$ expression cassette subcloned into the *Kpn*I site in the mutated *URA3* gene	This study
pHV13	pHV11 containing the $PGK1_p$-*mae2*-$PGK1_t$ expression cassette subcloned into the *Not*I site in the mutated *URA3* gene	This study

by *Kpn*I digestion, filled to blunt-ends with Klenow enzyme and religated to yield pHV9. A 944 bp upstream *URA3* fragment was PCR amplified from *S. cerevisiae* S92 genomic DNA using primer set 5'-XBASFRURA3 and 3'-URA3KPN (Table 2), while a 959 bp downstream *URA3* fragment was PCR amplified using primer set 5'-KPNNOTURA3 and 3'-URA3SFRXBA.

Both the upstream and downstream *URA3* fragments were digested with *Kpn*I and fused by T_4 DNA ligation. The resulting linear product, which was isolated after 1% agarose gel electrophoresis and purified by the High Pure Gel Extraction Kit, served as template for PCR amplification with primer set 5'-XBASFRURA3 and 3'-URA3SFRXBA. The modified *URA3* fragment containing unique cloning sites (*Kpn*I, *Not*I) and excision sites (*SrfI* and *Xba*I) was subcloned in the *Xba*I restriction site of pHV9, resulting in pHVJH1 (Fig. 1).

Construction of the expression vectors and pHV3 and pHV7 (Table 1) was previously described (Volschenk *et al.*, 1977a,b; Volschenk *et al.*, 2001). The $PGK1_p$-*mae1*-$PGK1_t$ expression cassette was PCR amplified using primer set 5'-KPNPGK and 3'-PGKKPN with plasmid pHV3 as template, while primers 5'-NOTPGK and 3'-PGKNOT were used for PCR amplification of the $PGK1_p$-*mae2*-$PGK1_t$ expression cassette from plasmid pHV7 (Volschenk *et al.*, 2001). The $PGK1_p$-*mae1*-$PGK1_t$ PCR product was subcloned as a *Kpn*I fragment into pHVJH1 to yield pHV11 (Fig. 2). Similarly, the $PGK1_p$-*mae2*-$PGK1_t$ PCR product was subcloned as a *Not*I fragment into pHV11 to yield pHV13. *SrfI* digestion of pHV13 resulted in the excision of a linear $PGK1_p$-*mae1*-$PGK1_t$ -$PGK1_p$-*mae2*-$PGK1_t$ fragment flanked by ca. 500 to 600 bp *URA3* sequences, which excludes any vector-derived DNA sequences.

Phleomycin and geneticin resistance of industrial wine yeast strains

The minimum inhibition concentration (MIC) of phleomycin and geneticin for *S. cerevisiae* S92 was determined. Yeast cells were cultured overnight in 10 mL YEG broth and plated with or without electroporation (in the absence of any DNA) onto YEG plates with Pastagar B (Difco Laboratories, Detroit, MI), containing a range of 5 µg/mL to 500 µg/mL phleomycin or geneticin. A minimum concentration of 100 µg/mL geneticin was required for complete inhibition *S. cerevisiae* S92 prior to electroporation. However, electroporated cells of *S. cerevisiae* S92 cells gave rise to background colonies (false positives) even at a concentration of 500 µg/mL geneticin. The minimum inhibitory concentration of phleomycin was determined at 250 µg/mL for electroporated cells of *S. cerevisiae* S92.

Adaptation of GMIA media for optimised malo-ethanolic phenotype selection

A plate assay method was developed to simplify the selection of positive transformants with a malo-ethanolic phenotype after electroporation and integration. The malo-ethanolic plate assay was also used to determine the dominance of the malo-ethanolic recombinant yeast strain during the subsequent wine fermentations. The Glucose-Malate-Indicator Agar (GMIA) selection medium was previously developed for the malo-ethanolic yeast *S. pombe* (Osothsilp *et al.*, 1986). The plates produce blue colonies with a surrounding blue halo when L-malic acid in the media is degraded by *S. pombe* due to a shift in pH (pH 3.3 to 5.2) when L-malic acid is converted to pyruvic acid. Initial attempts with a recombinant strain of *S. cerevisiae* containing the malo-ethanolic genes on a multi-copy plasmid (Volschenk *et al.*, 2001) did not produce a clear

TABLE 2

List of PCR primers used in this study to construct the linear integration cassette containing the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ expression cassettes flanked by *URA3* sequences.

Primer name	Primer sequence
5'-XBASFRURA3	5'-GATC***TCTAGA***GCCCGGGCAACGGTTCATCATCTCATGGATCTGC-3'
3'-URA3KPN	5'-GATC**GGTACC**TACTTCTTCCGCCGCCTGCTTCAAACCGCT-3'
5'-KPNNOTURA3	5'-GATC**GGTACC***GCGGCCGC*ACAAAGGAACCTAGAGGCCTTTTGATGTTAG-3'
3'-URA3SFRXBA	5'-GATC***TCTAGA***GCCCGGGCTACACCAGAGATACATAATTAGATAT-3'
5'-KPNPGK	5'-GATC**GGTACC**AACCTTTCTAACTGATC-3'
3'-PGKKPN	5'-GATC**GGTACC**AAGCTTTAACGAACGCA-3'
5'-NOTPGK	5'-GATC*GCGGCCGC*AACCTTTCTAACTGATCTATCCAAAACTG-3'
3'-PGKNOT	5'-GATC*GCGGCCGC*AAGCTTTAACGAACGCAGAATTTTCG-3'
5'-mae1	5'-GATCGAATTCATGGGTGAACTCAAGGAAAT-3'
3'-mae1	5'-GATCAGATCTTTAAACGCTTTCATGTTCACT-3'
5'-mae2	5'-GATCGAATTCATGCCTGCAGGAACCAAAGAA-3'
3'-mae2	5'-GATCCTCGAGTTATACAAAAGGCTTGTATTC-3'
5'-mae1DIG	5'-CTTTCAATATCCACGTTCATCGACA-3'
3'-mae1DIG	5'-GAGACAGTAACACCAAGCAGCAAGA-3'
5'-mae2DIG	5'-GAACCAAAGAACAAATCGAGTGTCC-3'
3'-mae2DIG	5'-GAGAACAATGGGCAAGAATCGATTA-3'

TCTAGA = *Xba*I, GCCCGGGC = *SrfI*, **GGTACC** = *Kpn*I, *GCGGCCGC* = *Not*I.

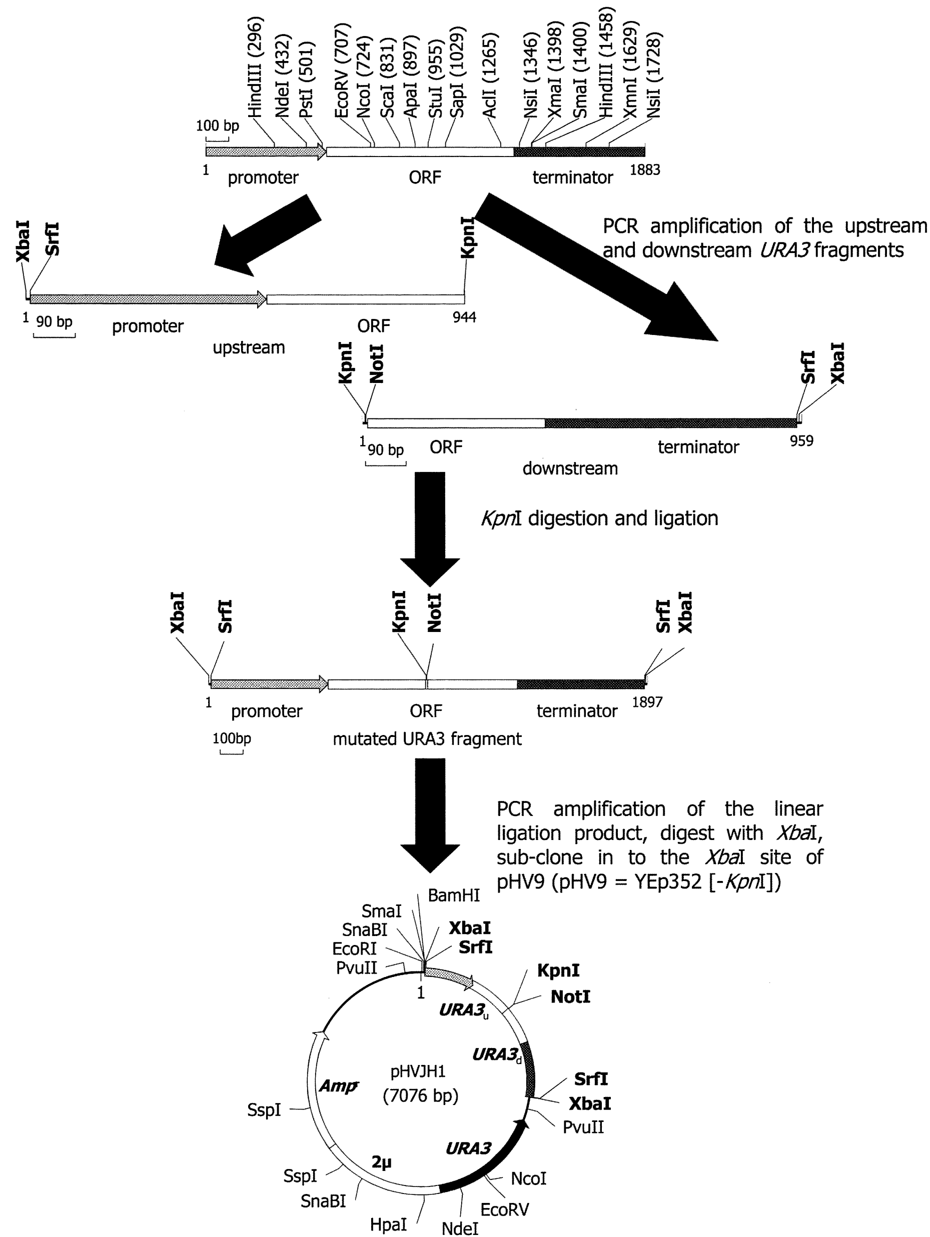

FIGURE 1

Construction of plasmid pHVJH1 by subcloning an upstream and downstream region of the *URA3* gene synthesised by PCR amplification to create unique restriction sites (*Kpn*I and *Not*I) for subcloning and excision sites (*Srf*I and *Xba*I).

phenotype for L-malic acid degradation on the GMIA plates. The original GMIA medium was therefore modified to contain 0.17% Yeast Nitrogen Base (Difco Laboratories, Detroit, MI), 0.5% $(NH_4)_2SO_4$, 10% glucose (to simulate glucose levels in grape must), 10% L-malic acid, 0.01% bromocresol green and 2% Noble agar (Difco Laboratories, Detroit, MI), instead of the Bacto-agar. The pH of the optimised GMIA media was adjusted to 3.3 with KOH.

Co-transformation and integration of *mae1* and *mae2* genes in *S. cerevisiae* S92

Integration of the *mae1* and *mae2* genes into the genome of commercial wine yeast strains was obtained by co-transformation of the linear *URA3*-flanked $PGK1_p$-*mae1*-$PGK1_t$-$PGK1_p$-*mae2*-$PGK1_t$ integration cassette and plasmid pUT332, which contains the *Tn5ble* gene for selection of phleomycin resistance (Gatignol *et al*., 1990; Wenzel *et al*., 1992). Initial screening on phleomycin-containing media was required to select for successful transformation and to minimise the number of colonies to be screened for the malo-ethanolic phenotype.

An adapted electroporation method was used in this study for the transformation of industrial wine yeast strains. Yeast cells were pre-cultured overnight in 10 mL YPD at 30°C followed by 500 mL YPD in a 2 L flask to an optical density at 600 nm (OD_{600}) of 0.1. The culture was shaken vigorously at 30°C until an OD_{600} of 1.3 to 1.5 was reached. Yeast cells were harvested by

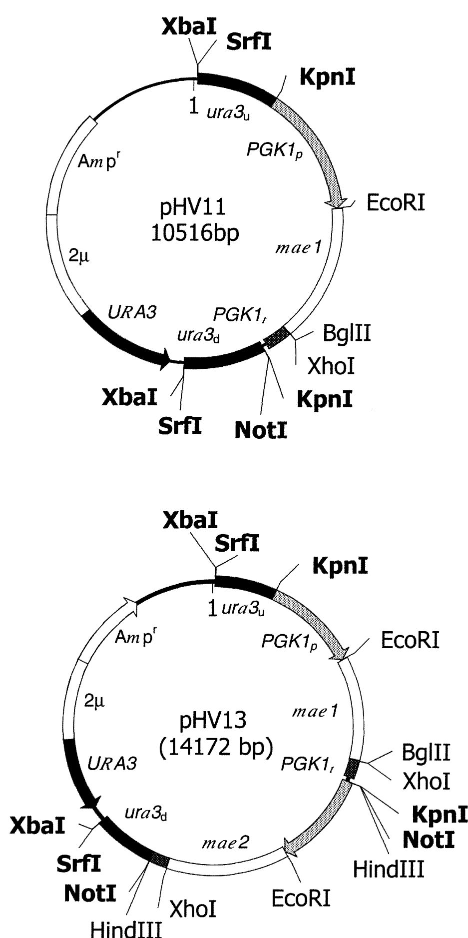

FIGURE 2

Plasmid maps of pHV11 containing the *PGK1*p-*mae1*-*PGK1*t expression cassette and plasmid pHV13 containing both the *PGK1*p-*mae1*-*PGK1*t and *PGK1*p-*mae2*-*PGK1*t expression cassettes. *Srf*I digestion of pHV13 yielded a linear integration cassette without any vector, bacterial or other foreign DNA sequences that were used for co-transformation with plasmid pUT332.

centrifugation at 4000 x g at 4°C and re-suspended in 80 mL double-distilled water (sterilised). While swirling, 10 mL 10 X TE buffer (pH 7.5) was added, followed by 10 mL 1 M LiOAc. After incubation for 45 min at 30°C with gentle agitation, 2.5 mL fresh 1 M DTT was added to the yeast suspension, while swirling, with a continued incubation for 15 min at 30°C with gentle agitation. The yeast suspension was subsequently diluted to a volume of 500 mL with double-distilled water, washed and concentrated three times at 4000 x g, 4°C. Cell pellets were re-suspended first in 250 mL ice-cold double-distilled water, then in 30 mL ice-cold 1 M sorbitol and finally in 0.5 mL ice-cold 1 M sorbitol. This yielded a final volume of 1 – 1.5 mL cells with an approximate OD_{600} of 200. After the cell pellet was re-suspended, 40 µL of the concentrated yeast cells was mixed with 5 µL DNA in a sterile, ice-cold 1.5 mL tube. A 10:1 molar ratio of linear:plasmid DNA was used, with ideally 50 ng of pUT332 and an appropriate 10-fold molar increase of linear DNA. The cell-DNA mixtures were transferred to an ice-cold 0.2 cm gap electroporation cuvette (Biorad, South Africa) and subjected to a pulse of 1.5 kV, 25 mF and 200 ohms (Gene Pulser II Electroporator, Biorad, South Africa). Immediately after the pulse was administered, 1 mL ice-cold YPD (1% yeast extract, 2% peptone and 2% glucose) was added to the cuvette, followed by a gentle mix for 2 to 4 h at 30°C. Aliquots of 250 µL yeast suspension were spread directly onto YEG plates containing 250 µg/mL phleomycin. Transformants were incubated for 3 to 4 days at 30°C. Putative transformants were inoculated in 10 mL YPD (non-selective conditions) and cultured successively for > 200 generations at 30°C to cure the yeast of plasmid pUT332. After the loss of plasmid pUT332 was confirmed on phleomycin media (data not shown), transformants were streaked onto modified GMIA plates.

PCR confirmation of integration and Southern blotting

Initial proof of the integration of the linear *PGK1*p-*mae1*-*PGK1*t -*PGK1*p-*mae2*-*PGK1*t fragment was obtained through PCR amplification of the entire *mae1* and *mae2* open reading frames using primer sets 5'-mae1/3'-mae1 and 5'-mae2/3'-mae2, respectively (Table 2). Integration of the linear *PGK1*p-*mae1*-*PGK1*t-*PGK1*p-*mae2*-*PGK1*t fragment in the genomic *URA3* locus was confirmed through Southern blot analysis. Standard procedures (Ausubel *et al.*, 1995) were used to prepare the gel for Southern blotting and to transfer the DNA to a positively charged nylon membrane (Roche Diagnostics, Germany). Genomic DNA was isolated from *S. cerevisiae* (Hoffman & Winston, 1987), digested with *Hpa*I and separated on a 1% agarose gel. An internal 944 bp *URA3* fragment corresponding to the upstream *URA3* region used for construction of the linear integration cassette was DIG-labelled (PCR Probe Synthesis Kit, Roche Diagnostics, Germany) using primer set 5'-XBASFRURA3 and 3'-URA3KPN. The presence of the *URA3* gene was visualised with the Chemiluminescent Detection Kit (Roche Biochemicals, Germany).

Malo-ethanolic fermentation in grape must

Synthetic grape must

The host yeast strain, *S. cerevisiae* S92, and three transformants (MEF2) containing the integrated *mae1* and *mae2* genes were inoculated at 2 x 10^6 cells /mL into duplicate sets of 200 mL synthetic grape must in 250 mL Erlenmeyer flasks (Denayrolles *et al.*, 1995). The synthetic grape must contained 0.94% L-malic acid (Sigma, St. Louis, MO) and the pH was adjusted with 1 M KOH to 3.3. Fermentations were carried out at 20°C without shaking and sealed with fermentation caps filled with 2.5% SO_2 solution for approximately 15 days. Growth of yeast cells was monitored spectrophotometrically at OD_{600}.

Small-scale grape must fermentation

Small-scale fermentations were also performed in Chardonnay (3 g/L L-malic acid, pH 3.40), Cabernet Sauvignon (2.5 g/L L-malic acid, pH 3.77), Colombard (4.5 g/L L-malic acid, pH 3.42) and Ruby Cabernet (3.5 g/L L-malic acid, pH 3.54) grape musts. To ensure dominance in the final yeast population, the host and the malo-ethanolic recombinant strain, MEF2, containing the integrated *mae1* and *mae2* genes were inoculated at 2 x 10^6 cells/mL into 400 mL must in 500 mL flasks and incubated at 20°C without shaking. The flasks were sealed with fermentation caps filled with 2.5% SO_2 solution. White and red grape musts were supplemented with 50 ppm and 30 ppm SO_2 respectively, while 0.075% diammonium phosphate was added to all flasks before inoculation to ensure a sufficient nitrogen source during fermentation. The weight of the fermentation flasks was measured at regular intervals as an indication of fermentation speed by indi-

rectly measuring CO_2 production and evaporation, while the presence and dominance of the recombinant malo-ethanolic strains were verified by regular serial dilutions of the fermenting juice and screening on the optimised GMIA media.

Large-scale vinification for sensory evaluation

Chardonnay grapes (23.7°B) were harvested during the 2001 season, de-stemmed, crushed and pressed. The must was treated with 50 mg/L SO_2 and allowed to settle overnight. Chemical analysis indicated that the Chardonnay juice contained 2.97 g/L L-malic acid, a pH of 3.53 and a total acidity of 6.27 g/L. Similarly, the Cabernet Sauvignon grapes (21.8°B) were de-stemmed and crushed, treated with 30 mg/L SO_2 and divided into 12 lots of 15 L each, followed by direct inoculation with yeasts as described above. The Cabernet Sauvignon must contained 3.5 g/L L-malic acid, a pH of 3.77 and a total acidity of 7.16 g/L. After three days of skin contact, the must was pressed and returned to fermentation flasks for further alcoholic fermentation.

The Chardonnay and Cabernet Sauvignon juice was divided into 12 lots of 15 L each for three repetitions of two different treatments, i.e. (i) inoculation with the host yeast strain, *S. cerevisiae* S92, as a control fermentation, or (ii) three positive transformants containing the integrated malo-ethanolic genes. The yeast was inoculated at a final concentration of 2 X 10^6 cells/mL to ensure dominance in the final yeast population. The presence and dominance of the recombinant malo-ethanolic strains were confirmed at the beginning, halfway mark and at the end of alcoholic fermentation by serial dilutions of the fermenting juice and screening on the optimised GMIA media. Fermentations in Chardonnay must were carried out at 15°C, while Cabernet Sauvignon must was fermented at 23°C. After alcoholic fermentation was completed, one set of control wines (*S. cerevisiae* S92) from both Chardonnay and Cabernet Sauvignon was inoculated with Viniflora Oenos (Chris Hansen, Denmark) for MLF according to the manufacturer's recommendations. All other wines were decanted and treated with 30 mg/L SO_2 and stored at 0°C for seven days for cold stabilisation. An experienced panel of 15 judges performed organoleptic evaluation of the Chardonnay wine six months after bottling. A ranking method was used to determine differences between the treatments and statistical significance was determined according to Amerine & Roessler (1976).

Chemical analysis

The concentrations of L-malic acid, D-glucose, glycerol and ethanol were determined using enzymatic assays (Roche Diagnostics, Germany). In-depth analysis of large-scale fermented wines were done by Capillary Electrophoresis (HP3D CE system, Hewlett-Packard) and GrapeScan 2000 (FOSS Electric A/S, Denmark) to determine glucose, fructose, glycerol, ethanol, tartaric, malic, citric, succinic, acetic and lactic acid concentrations. CE analysis was carried out with a diode array detector. The CE detector wavelength was fixed at 200 nm with 350 nm as the reference wavelength. A bare silica capillary with an internal diameter of 50 µm (total length = 80.5 cm and effective length = 72 cm) was used for wine analysis and samples were injected hydrodynamically (50 mbar for 2 sec). A constant voltage of -25 kV was applied during the separation run and the temperature of the column was set at 25°C. The "HP organic acid buffer" was used as the separation buffer. Wine samples were centrifuged (8 min x 12 000 rpm) before diluting them 20-fold in MilliQ water. A standard solution of L-tartaric acid (60 mg/L), L-malic acid (40 mg/L), citric acid (20 mg/L), succinic acid (20 mg/L), acetic acid (20 mg/L) and lactic acid (20 mg/L) was prepared freshly and run between samples to create valid calibration curves for each component. HP Chemstation Software was used to calculate the concentrations of L-tartaric, L-malic, citric, succinic, acetic and lactic acids in the wine using data obtained from the standard and sample runs. The commercial calibration for the Grapescan 2000 was verified and adjusted to South African wines and conditions to ensure the correct intercepts. Standard methods were used to confirm the results obtained for residual sugar, final ethanol content, pH, total and volatile acidity of the finished wines (Ough & Amerine, 1987).

RESULTS AND DISCUSSION

Transformation of *S. cerevisiae* S92 with integration cassette

Electroporation of competent cells of *S. cerevisiae* with the linear malo-ethanolic integration cassette and pUT332 resulted in 100 to 200 phleomycin-resistant transformants per µg of linear DNA. The optimised GMIA medium allowed for the effective screening of the transformants with the integrated malo-ethanolic cassette after the initial screening of transformants on phleomycin-containing medium. Phleomycin-resistant transformants were transferred to GMIA plates and screened accordingly for the malo-ethanolic phenotype. Transformants with the ability to degrade L-malic acid appeared as blue-coloured colonies that could be easily distinguished from transformants lacking the malo-ethanolic phenotype (yellow/brown colonies).

To cure the transformants of the pUT332 plasmid, the transformants were individually picked and cultured in non-selective conditions (YPD broth) for more than 200 generations to obtain a phleomycin-sensitive phenotype that corresponded to the loss of pUT332 carrying the resistance marker gene, *Tn5ble*. Transformants cured of pUT332 were subsequently spotted onto GMIA media to confirm the presence of a malo-ethanolic phenotype. Colonies with a positive malo-ethanolic phenotype were re-inoculated into non-selective media and repeated in triplicate on GMIA media to determine the stability of the malo-ethanolic phenotype. The malo-ethanolic phenotype was considered to be stable in transformants if less than 1/10,000 revertant colonies appeared after each round of non-selective growth. Genetically engineered yeasts produced in this manner should be more acceptable for industrial application, since no antibiotic resistance markers are present in the recombinant yeast strain.

PCR amplification and Southern blot analysis of integration

The presence of the *mae1* and *mae2* open reading frames in the genome of *S. cerevisiae* S92 transformants (MEF) was confirmed by PCR amplification of a 1317 bp and 1698 bp fragment, corresponding to the complete open reading frame of the *mae1* and *mae2* genes, respectively (Fig. 3A). The host strain (wt) did not yield any PCR products under the same conditions.

The PCR product yield was significantly higher in transformants where multiple integrations of the malo-ethanolic cassette occurred (MEF1) compared to the single integration events (MEF2). Integration of the malo-ethanolic cassette in the *URA3* gene was also confirmed with Southern blot analysis that clearly

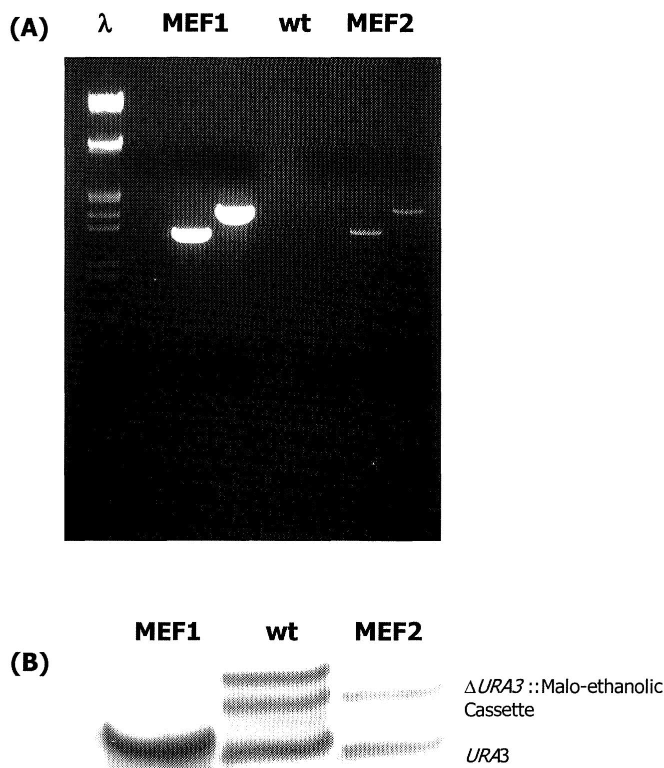

FIGURE 3

(A) PCR amplification of the *mae1* (1317 bp) and *mae2* (1698 bp) open reading frames using genomic DNA from *S. cerevisiae* S92 and selected transformants as template. (B) Southern blot results showing single (MEF2) or multiple (MEF1) integration of the malo-ethanolic cassette in the *URA3* locus. wt = host strain *S. cerevisiae* S92.

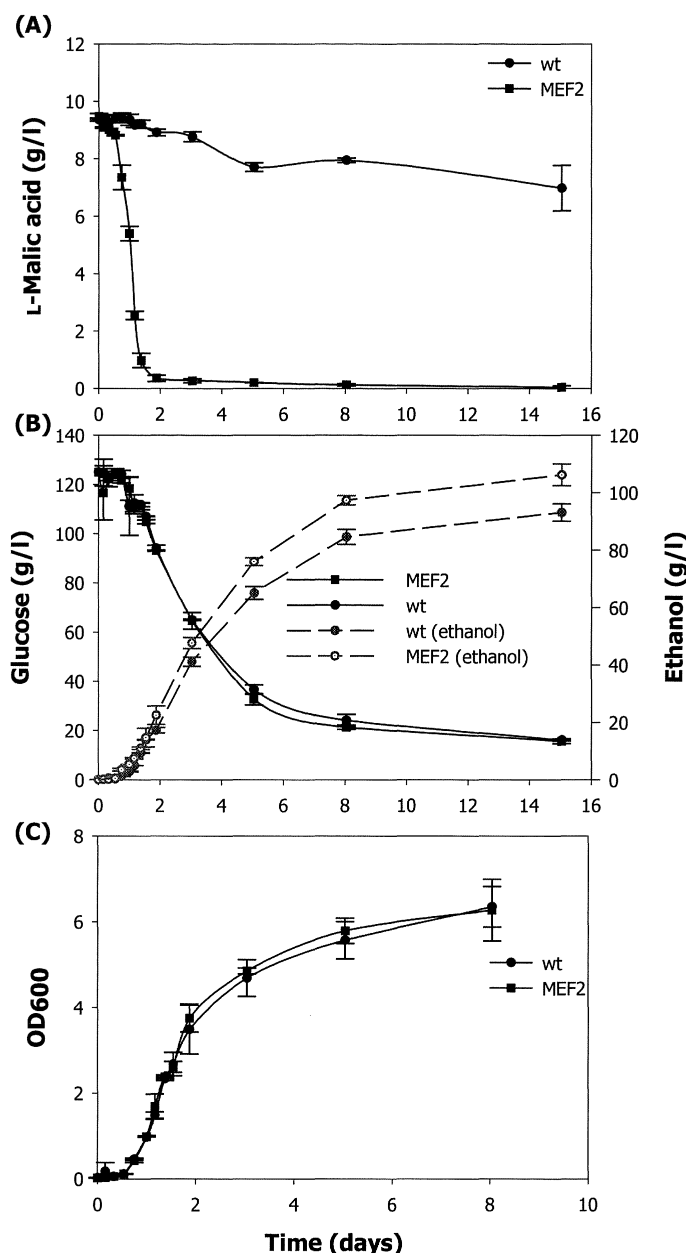

FIGURE 4

(A) L-malic acid degradation by MEF2 compared to the control yeast (*S. cerevisiae* S92, wt); (B) Glucose utilisation and ethanol production by the MEF2 strain compared to the control yeast during alcoholic fermentation, and (C) growth curve of the malo-ethanolic yeast (MEF2) and control strain in synthetic grape must as measured by cell density at OD_{600}.

demonstrated single (MEF2) or multiple integration (MEF1) events into the *URA3* locus (Fig. 3B). MEF2 transformants containing a single integration of the malo-ethanolic cassette were used for subsequent fermentation and sensory evaluation experiments.

Malo-ethanolic fermentation in synthetic and actual grape must

Rapid and efficient degradation of ca. 9.5 g/L L-malic acid within 5 days was obtained in synthetic grape must during small-scale fermentations by a recombinant strain of *S. cerevisiae* S92 (MEF2), which contains a single integrated copy of the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*-$PGK1_t$ expression cassettes (Fig. 4A). The host strain (*S. cerevisiae* S92) showed no significant degradation of L-malic acid during the first 5 days of fermentation, but after 15 days almost 32% of the total L-malic acid was degraded by this strain. This reduction in L-malic acid concentration by the control yeast strain is not ascribed to the active metabolism of L-malic acid by the yeast cells, but rather to the release of intracellular enzymes, i.e. malate dehydrogenases and the native malic enzyme during yeast autolysis at the late stationary phase of fermentation. Comparison of the growth rate and the rate of glucose consumption between the MEF2 and host strain showed no significant aberrations (Fig. 5B and C). This suggested that the introduction of the heterologous genes had no adverse effect on the recombinant yeast's growth and fermentation capacity.

During fermentative sugar metabolism, pyruvic acid is further decarboxylated to acetaldehyde by pyruvate decarboxylase and subsequently reduced to ethanol by alcohol dehydrogenase. Theoretically, the introduction of an efficient malo-ethanolic pathway in yeast should contribute additional pyruvic acid to the existing intracellular pool, promoting the production of elevated levels of ethanol. As previously reported for laboratory strains

(Volschenk *et al.*, 2001), the MEF2 strain consistently produced higher levels of ethanol relative to the host strain (Fig. 4B), confirming that the two *S. pombe* genes enabled cells of *S. cerevisiae* to metabolise the extra-cellular L-malic acid to ethanol under fermentative conditions.

The ability of the malo-ethanolic MEF2 wine yeast strain to degrade L-malic acid during alcoholic fermentation was also investigated during small-scale fermentations in Cabernet Sauvignon, Chardonnay, Colombard and Ruby Cabernet grape musts (Fig. 5A). Rapid and complete degradation of extracellular L-malic acid degradation was observed for MEF2 within 1, 1.5, 2 and 5 days in Cabernet Sauvignon, Ruby Cabernet, Colombard and Chardonnay grape musts, respectively. The host yeast strain did not contribute significantly to the degradation of L-malic acid

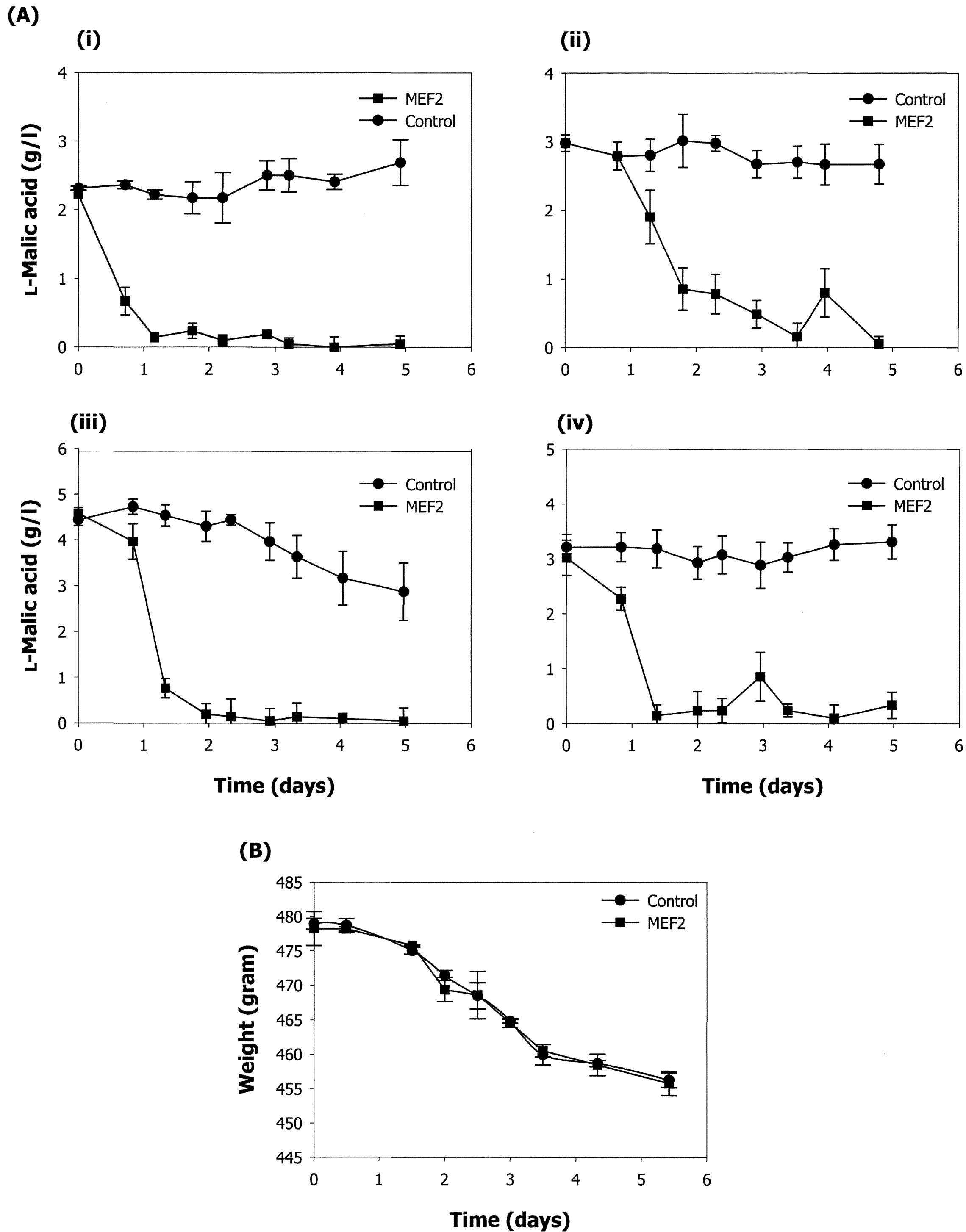

FIGURE 5

(A) Malo-ethanolic fermentation in (i) Cabernet Sauvignon, (ii) Chardonnay, (iii) Colombard and (iv) Ruby Cabernet grape musts by MEF2 and the control yeast strain *S. cerevisiae* S92. (B) Fermentation rate of MEF2 and the control yeast strain in Chardonnay grape must as measured by the loss of CO_2 during alcoholic fermentation.

in the corresponding control fermentations. Furthermore, the fermentation rate measured as the loss of weight (CO_2 evaporation) was almost identical for MEF2 and the host yeast strain (Fig. 5B), confirming that the expression of integrated *mae1* and *mae2* genes did not adversely affect the alcoholic fermentation capacity in the recombinant yeast. Serial dilutions of the fermenting juice followed by plating on optimised GMIA agar (results not shown) consistently indicated that the population of recombinant MEF2 yeast showed rapid growth to reach 10^8 and 10^9 cells per mL and dominance in the fermentation ($\geq$ 85% of total yeast population) after 2 days and throughout the remainder of alcoholic fermentation.

Effect of malo-ethanolic fermentation on organoleptic quality of wine

The ability of the MEF2 recombinant strain to produce a wine of quality was also evaluated during larger-scale vinification of Chardonnay and Cabernet Sauvignon grape musts. Standard winemaking practices were employed during the vinification, including the inoculation of the malolactic bacterium *O. oeni*, after alcoholic fermentation with *S. cerevisiae* S92 was completed. The malo-ethanolic yeast (MEF2) efficiently degraded all the L-malic acid in both Chardonnay and Cabernet Sauvignon grape musts, whereas the host strain, *S. cerevisiae* S92, had little effect on the L-malic acid concentration (Fig. 6). Serial dilutions of the fermenting juice followed by plating on optimised GMIA agar (results not shown) indicated that the recombinant strain of *S. cerevisiae* S92 (MEF2) became the dominant yeast species ($\geq$ 88% of total yeast population) after 1 and 2 days in Chardonnay and Cabernet Sauvignon grape musts, respectively, and maintained dominance throughout the remainder of alcoholic fermentation, confirming that the reduction in the L-malic acid is due the presence of the recombinant yeast strain and not by non-*Saccharomyces* strains also present in the juice.

The decrease in L-malic acid concentration also correlated with the decrease in total acidity (Table 3). In the wine fermented with MEF2, total acidity decreased by 2.3 g/L in agreement with complete L-malic acid decomposition. Total acidity was decreased by 1.34 g/L in the wine that underwent MLF after alcoholic fermentation by *S. cerevisiae* S92, and by 0.54 g/L for wine produced by *S. cerevisiae* S92 without MLF. The decrease in acidity was also reflected in the pH of the different wines; the pH of the wine fermented with MEF2 increased by 0.46 units, whereas fermentation with *S. cerevisiae* S92 with and without MLF resulted in a pH increase of only 0.28 and 0.16 units, respectively. Chemical analysis of the final wines indicated no significant changes to the other organic acids in the wine fermented with MEF2 and the control yeast. The tartaric, citric and succinic acid concentrations remained relatively unchanged for the different treatments (Table 3). The concentration of lactic acid remained relatively constant for the control and MEF2 wine, while a significant increase in lactic acid could be seen in the wine that underwent MLF. Furthermore, volatile acidity as measured by the acetic acid concentration, was slightly increased in the wine fermented with MEF2, but still within the threshold value for acetic acid in wine compared to the control yeast fermentation.

Comparison of the final ethanol values of the wines indicated an insignificant increase in ethanol concentration of the wine fermented with MEF2 in relation to the control wine fermented with

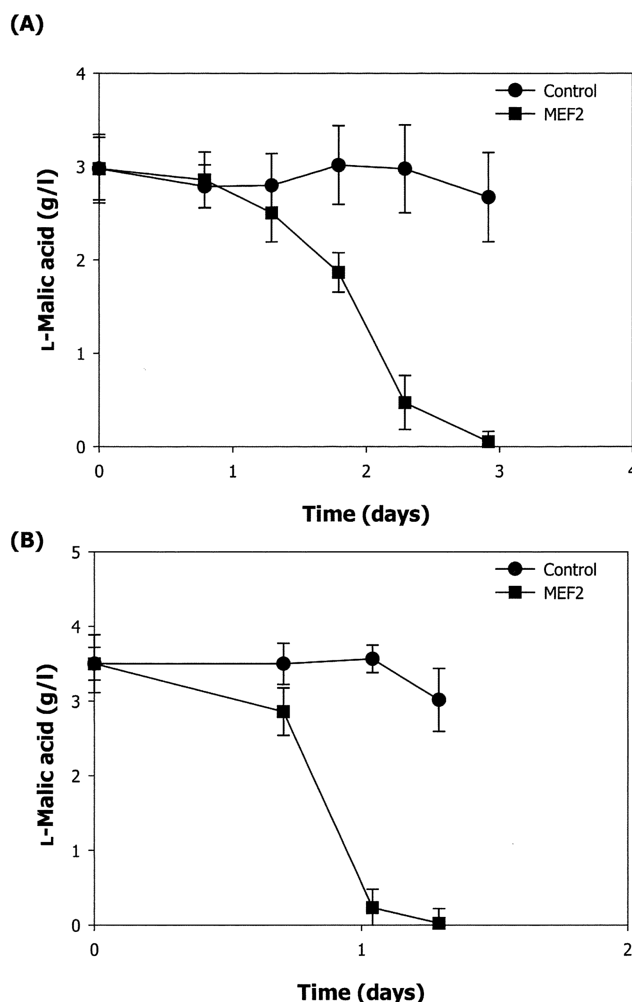

FIGURE 6

L-malic acid degradation during larger-scale fermentation of (A) Chardonnay and (B) Cabernet Sauvignon grape must with the recombinant MEF2 yeast containing the integrated malo-ethanolic expression cassette. The control fermentation was performed using the host yeast strain, *S. cerevisiae* S92.

S. cerevisiae S92. Glycerol production in yeast acts as a mechanism to rectify a possible NAD^+/NADH imbalance during yeast metabolism. Therefore, changes in the redox balance in yeast metabolism, such as during oxidative or osmotic stress conditions, are associated with changes in the amounts of glycerol produced by yeast (Nordström, 1968; Oura, 1977; Van Dijken & Scheffers, 1986; Larson *et al.*, 1998). Since the conversion of L-malic acid to pyruvic acid by the malic enzyme involves the reduction of NAD^+ to NADH, the level of glycerol in wine was also determined after alcoholic fermentation. Wine fermented by the MEF2 strain showed an increase of ca. 1 g/L glycerol relative to the wine fermented by the control yeast.

Organoleptic evaluations of the fermented Chardonnay wine indicated a significant difference between wines produced with *S. cerevisiae* S92, the MEF2 recombinant strain and wines that underwent MLF (Table 4). Based on the perceived aroma of the wines, the lowest score was obtained for the wine fermented by *S. cerevisiae* S92, while wine made by MEF2 scored the highest, even higher than the wine that underwent MLF. No off-flavours

TABLE 3
Chemical composition of fermented Chardonnay wine.

	Malic Acid (g/L)	Tartaric Acid (g/L)	Citric Acid (g/L)	Succinic Acid (g/L)	Acetic Acid (g/L)	Lactic Acid (g/L)	Glycerol (g/L)	Ethanol (g/L)	Residual Glucose (g/L)	Residual Fructose (g/L)	Total Acidity (g/L)	pH
Prior to fermentation	2.97								115	n.d.	6.27	3.53
	±0.03								±0.6		±0.02	±0.01
S. cerevisiae **S92**	2.82	1.26	0.36	0.26	0.38	0.09	6.5	14.3	0.44	3.13	5.73	3.69
	±0.2	±0.01	±0.01	±0.02	±0.01	±0.1	±0.1	±0.1	±0.6	±0.3	±0.6	±0.01
S. cerevisiae **S92+MLF**	0.29	1.16	0.24	0.27	0.38	1.32	6.4	14.48	1.31	1.75	4.93	3.81
	±0.2	±0.2	±0.1	±0.01	±0.02	±0.2	±0.1	±0.1	±0.5	±0.5	±0.4	±0.06
MEF2	0.22	1.47	0.44	0.27	0.56	0.13	7.2	14.6	1.24	2.64	3.97	3.99
	±0.1	±0.02	±0.18	±0.01	±0.01	±0.2	±0.2	±0.2	±0.4	±0.2	±0.2	±0.2

n.d. = not determined.
± = standard deviation.

TABLE 4
Results of the organoleptic evaluation of Chardonnay wine.

Treatment	Aroma order	Palate order
S. cerevisiae S92	3*	1**
S. cerevisiae S92+ MLF	2	2
MEF2	1**	3*

* – significant at $p<0.05$.
** significant at $p<0.01$.

were detected, while an increase in fruitiness was noted, possibly due to the absence of the masking effect of L-malic acid. These results indicate that the malo-ethanolic strain was more successful in producing fruity-floral aromas in wine, a definite advantage in the production of cultivars such as Muscat, Riesling, Sauvignon Blanc and Gewürztraminer. However, based on the perceived palate of the wine, the best results were obtained with wine fermented by the control yeast *S. cerevisiae* S92, while wine produced with MEF2 scored the lowest. The taste panel detected an imbalance in the acid:sugar ratio in the wine made by the malo-ethanolic yeast (MEF2). This could be expected since all the L-malic acid was completely removed from the wine and resulted in a sub-optimal final total acidity (Table 3). The Chardonnay and Cabernet Sauvignon musts used in this study were harvested from a warm-climate viticultural region and contained low levels of L-malic acid in the grape must, i.e. 2.97 and 3.5 g/L L-malic acid, respectively. Future evaluation of the malo-ethanolic yeast in high-acid wines from a cool-climate viticultural region will be required to determine the actual organoleptic influence of this recombinant yeast on wine.

CONCLUSIONS

In this study the commercial wine yeast *S. cerevisiae* S92 was successfully transformed through integration of a malo-ethanolic cassette containing the $PGK1_p$-*mae1*-$PGK1_t$ and $PGK1_p$-*mae2*- $PGK1_t$ linear integration cassette flanked by large *URA3* homologous sequences. A single genomic copy of the malo-ethanolic cassette in *S. cerevisiae* S92 was sufficient to yield a strong malo-ethanolic phenotype, i.e. the conversion of L-malic acid to ethanol, in the recombinant yeast in synthetic and grape must fermentations. Sensory evaluation and chemical analysis of a Chardonnay wine produced by the malo-ethanolic yeast indicated an improvement in wine aroma compared to the traditional MLF. Commercial availability of malo-ethanolic wine yeast will be especially beneficial in the production of fruity-floral wines and the de-acidification of high-acid wines in the cool-climate viticultural regions of the world.

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