MINISTRY OF EDUCATION
FEDERAL UNIVERSITY OF SERGIPE
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GRADUATE PROGRAM IN AGRICULTURE AND BIODIVERSITY

INTEGRATIVE BIOLOGY FOR PROSPECTING MARKERS OF GALL WASP RESISTANCE IN *Eucalyptus*

CRISLAINE COSTA CALAZANS

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Thesis submitted to the Federal University of Sergipe, in partial fulfillment of the requirements for the degree of “Doctor of Science”, of the Doctoral Course in Agriculture and Biodiversity, area of concentration Agriculture and Biodiversity

Primary advisor
Prof. Dr. Renata Silva-Mann

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CRISLAINE COSTA CALAZANS

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BIOLOGIA INTEGRATIVA PARA A PROSPECÇÃO DE MARCADORES DE RESISTÊNCIA À VESPA-DA-GALHA EM *Eucalyptus*

Thesis submitted to the Federal University of Sergipe, in partial fulfillment of the requirements for the degree of “Doctor of Science”, of the Doctoral Course in Agriculture and Biodiversity, area of concentration Agriculture and Biodiversity.

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To everyone who, at some point in my life, was there to reach out and encourage me with friendship and support, inspiring me to pursue this goal.

Dedication

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LIST OF ABBREVIATIONS AND SYMBOLS

AL	Alagoas
BA	Bahia
BLAST	Asic Local Alignment Search Tool
CERFLOR	Brazilian Forest Certification Program
ChEBI	Chemical Entities of Biologic Interest
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
ES	Espírito Santo
F	Phenylalanine
FPP	Trans-Farnesyl Diphosphate
FSC	Forest Stewardship Council
GMO	Genetically Modified
GO	Gene Ontology
GO	Goiás
He	Expected Heterozygosity
IPM	Integrated Pest Management
ISSR	Inter Simple Sequence Repeat
Li	*Leptocybe invasa*
LTR	Long Terminal Repeat
MA	Maranhão
MEP	Methylerythritol Phosphate
MFC	Mechanically Fibrillated Nano Cellulose
MG	Minas Gerais
MSA	Multiple Sequence Alignment
MT	Mato Grosso
MT	Mato Grosso do Sul
MVA	Mevalonate

NBARC	Nucleotide-Binding ARC-Domain
Om	*Ophelimus maskelli*
PA	Pará
PAL	Phenylalanine Ammonia-Lyase
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PE	Pernambuco
PI	Piauí
PPi	Pyrophosphate
PR	Paraná
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RNA	Ribonucleic Acid
RS	Rio Grande do Sul
SC	Santa Catarina
SE	Standard Error
SE	Sergipe
SP	São Paulo
SSR	Simple Sequence Repeats
TE	Tris + EDTA
TE	Transposable Elements
TIR-NBS-LRR	Tollinterleukin-1-Receptor-Nucleotide Binding Signal Leucine-Rich Repeat
TO	Tocantins
uHe	Unbiased Expected Heterozygosity
UPGMA	Unweighted Pair Group Method With Arithmetic Mean
URP	Universal Rice Primers
W	Tryptophan
WIPO	World Intellectual Property Organization
Y	Tyrosine

RESUMO

CALAZANS, Crislaine Costa Calazans. **Biologia integrativa para a prospecção de marcadores de resistência à vespa-da-galha em *Eucalyptus*.** São Cristóvão: UFS, 2023. 100p. (Tese – Doutorado em Agricultura e Biodiversidade).*

No Brasil, a vespa-da-galha, *Leptocybe invasa*, causa prejuízos à produção de *Eucalyptus*. Esta tese teve como objetivo explorar o conhecimento sobre *L. invasa* em genótipos de *Eucalyptus*, identificar genes e vias metabólicas relacionadas às interações planta-inseto, bem como caracterizar genótipos quanto à resistência à vespa-da-galha, utilizando elementos transponíveis e marcadores ISSR, buscando contribuir para o controle dessa praga. O estudo foi dividido em três etapas: i) meta-análise de associações gênicas para combater *L. invasa*; ii) investigação *in silico* e iii) Análise fenotípica e genotípica de genótipos de *Eucalyptus*. Para a meta-análise, utilizou-se como palavras-chave "*Leptocybe invasa*" OR "gall wasp *Eucalyptus*" em artigos nas bases de dados científicas *Scopus* e *Web of Science*, com análise facilitada pelo pacote R Bibliometrix. Associou-se as informações de anotação do *E. grandis* do Phytozome v7.0. Quanto a vias metabólicas empregou-se o banco de dados *Plant Reactome Gramene Pathways* e as inovações tecnológicas na forma de patentes foram prospectadas com o software *Orbit Intelligence*. Por meio de análises *in silico*, identificou-se sequências conservadas de elementos transponíveis e do genoma de *Eucalyptus* spp. Para distinguir genótipos resistentes e suscetíveis com base nos danos causados pela vespa-da-galha, empregou-se análise de imagens capturadas no equipamento Groundeye®. A discriminação dos genótipos em resposta a *L. invasa* foi feita com sequências de elementos transponíveis e marcadores ISSR. Na meta-análise incluiu-se 234 documentos, sendo 116 de *Web of Science* e 118 de *Scopus*. A análise das vias metabólicas revelou uma via associada ao gene *Eucgr.B02310*, relacionado a presença de terpenos. A maioria das patentes prospectadas trata de pesticidas para o controle de *L. invasa*. Na análise *in silico* identificou-se regiões conservadas no genoma de *Eucalyptus* spp., associadas à produção de metabólitos secundários, como terpenos. A análise de fenotípica foi eficiente na distinção entre os genótipos suscetíveis e resistentes à vespa-da-galha. Os elementos transponíveis e marcadores ISSR produziram fragmentos suficientes para diferenciar genótipos resistentes dos suscetíveis. Esses resultados contribuem para o manejo de *L. invasa* em *Eucalyptus* e evidênciam a importância da combinação de abordagens biológicas, químicas e genéticas para este fim.

Palavras-chave: bioinformática, *in silico,* ISSR, *Leptocybe invasa,* terpenos, transposons.

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* Comitê Orientador: Profa. Dra. Renata Silva-Mann– UFS (Orientadora).

ABSTRACT

CALAZANS, Crislaine Costa. **Integrative biology for prospecting markers for gall wasp resistance in *Eucalyptus.*** São Cristóvão: UFS, 2022. 100p. (Thesis - Doctoral Course in Agriculture and Biodiversity).*

In Brazil, the gall wasp, *Leptocybe invasa,* causes damage to *Eucalyptus* production. The objectives of this thesis were to study *L. invasa* in *Eucalyptus* genotypes and identify genes and metabolic pathways related to plant-insect interactions. In addition, in an effort to contribute to the control of this pest, genotypes were characterized in terms of resistance to the gall wasp, using transposable elements and ISSR markers. The study was divided into three stages: i) meta-analysis of gene associations to combat *L. invasa*; ii) *in silico* analysis; and iii) phenotypic and genotypic analysis of *Eucalyptus* genotypes. For the meta-analysis, the search terms "*Leptocybe invasa*" OR "gall wasp *Eucalyptus*" were used in articles from the scientific databases Scopus and Web of Science, using the R Bibliometrix package. Annotation information from *E. grandis* in Phytozome v7.0 was validated. Regarding metabolic pathways, the Plant Reactome Gramene Pathways database was employed and technological innovations in the form of patents were searched using the Orbit Intelligence® software. Conserved sequences of transposable elements of the *Eucalyptus* spp. genome were identified by *in silico* analyses. Resistant and susceptible genotypes were differentiated according to the gall wasp damage level, by means of image analysis with Groundeye® equipment. The genotype response to *L. invasa* was discriminated based on transposable element sequences and ISSR markers. The meta-analysis included 234 documents; 116 from the Web of Science and 118 from Scopus. Metabolic pathway analysis detected a pathway associated with the *Eucgr.B02310* gene, related to the presence of terpenes. Most of the surveyed patents deal with pesticide control of *L. invasa*. *In silico* analysis identified conserved regions in the *Eucalyptus* spp. genome, associated with the production of secondary metabolites such as terpenes. The phenotypic analysis effectively distinguished gall-wasp susceptible from -resistant genotypes. Transposable elements and ISSR markers generated sufficient fragments to differentiate resistant from susceptible genotypes. These results contribute to the management of *L. invasa* in *Eucalyptus* and highlight the importance of combining biological, chemical and genetic approaches for this purpose.

Keywords: Bioinformatics, *in silico*, ISSR, *Leptocybe invasa*, terpenes, transposable elements.

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* Advisory committee: Prof. Dr. Renata Silva-Mann – UFS (Primary advisor).

1. GENERAL INTRODUCTION

Afforestation is crucial in promoting global sustainability. With an estimated acreage of 294 million hectares worldwide, planted forests account for approximately 7% of the total forest area of the world (HUA et al., 2022). *Eucalyptus* is one of the most significant and globally most widely planted tree genera. Planted on approximately 25 million hectares around the world, *Eucalyptus* has come to play a key role in the forestry and timber industry (FAO, 2023).

Brazil is currently leading in in *Eucalyptus* cultivation, with a plantation area of 10 million hectares, which represents 76.2% of the total forest plantations in the country (IBGE, 2018; KUHLMANN; VAN DER MARK; BAFFONE SERGIO, 2019; RAJ; JHARIYA; BARGALI, 2016). The massive cultivation of *Eucalyptus* in Brazil clearly shows its significance in the forestry sector and the relevance of Brazilian *Eucalyptus* production at the global level.

Eucalyptus is an exotic species that can be exploited for multiple industrial applications and is being extensively exploited commercially. In the first place, *Eucalyptus* forests are planted to produce timber, fiber, energy, cellulose, paper, lumber and products with high domestic and international demand (DUBE et al., 2002; FLORÊNCIO; MARTINS; FAGUNDES, 2022). Additionally, *Eucalyptus* is an important source of biomass, in that forest thinning residues and sawdust are used for energy production, cellulose and other bio-based products (TREVORAH et al., 2018).

The remarkable expansion of *Eucalyptus* over the past century can be attributed to the socio-economic benefits of this crop, due to the biomass production, which is higher than that of most native Brazilian species (JALETA et al., 2016). However, the accidental introduction of the gall wasp, *Leptocybe invasa* Fisher & La Salle 2004 (Hymenoptera: Eulophidae: Tetrastichinae) into Brazil created a significant challenge for *Eucalyptus* production.

This insect species with endophytic oviposition induces the formation of galls on the leaves, petioles and young branches of plants (RINALDI et al., 2013). The damage it causes has a substantial impact on Brazilian silviculture, requiring sound data to determined effective control strategies that mitigate productivity losses in the forestry sector.

According to the international standards for the management of native and cultivated forests (Forest Stewardship Council), forest certification is not granted unless chemical insecticide application are strictly limited. As a result, alternative approaches are increasingly being sought. The results of previous efforts to control the gall-inducing pathogen by trapping and pesticide application have been unsatisfactory, mainly due to the small size of the causal agent, commonly known as the micro-wasp. This tininess is a challenge for effective field detection, as emphasized by Jacob et al. (2015).

However, a study in India demonstrated the effectiveness of biological control with parasitoids such as *Megastimus viggianii* Narendran & Sureshan (Hymenoptera: Torymidae); *Quadrastichus mendeli* Kim & La Salle (Hymenoptera: Eulophidae) in controlling *L. invasa* and preventing gall formation (YOUSUF, 2019). Although successful, biological control is associated with high costs and requires trained monitoring teams, making this alternative economically less attractive. Therefore, the pursuit of molecular solutions related to resistance is a viable alternative in insect control.

Traditionally, trees with improved traits have been developed in long-term breeding programs. Genomic and transcriptomic studies have emerged as promising tools in breeding programs, particularly the marker-assisted selection (NASCIMENTO et al., 2017). The discovery of new targets and genetic markers can significantly enhance the current germplasm of plants and facilitate the development of new cultivars, ultimately contributing to increased crop yield and quality while preserving the environment (COOPER et al., 2018).

Therefore, the identification and isolation of genes associated with gall wasp resistance are highly promising for the forestry sector. The overarching objectives of this research were a comprehensive analysis of *L. invasa* in *Eucalyptus* genotypes, of the underlying genes and metabolic pathways governing plant-insect interactions and an integrated analysis of the susceptibility of genotypes to gall wasp infestation based on image analysis, transposable elements and ISSR markers. This comprehensive approach seeks valuable insights to enhance gall wasp resistance strategies and optimize the forestry management of this pest.

2. LITERATURE REVIEW

2.1 *Eucalyptus*: a globally significant, versatile genus

The genus *Eucalyptus* L'Hér. (1788) belongs to the family Myrtaceae Juss. (1789). This diverse family comprises 134 genera and approximately 6,400 identified species and subspecies (GBIF, 2023). Myrtaceae species are typically trees or shrubs, with glabrescent or densely pubescent branches that can be gray or reddish, with simple, opposite leaves that have entire margins, brochidodromous venation and glands (AMORIM et al., 2022).

Six new species have been included in the Myrtaceae family, in the genera *Acca*, *Corymbia* and *Eucalyptus* (LUCAS et al., 2023). *Eucalyptus*, along with *Pinus* and *Cunninghamia*, is one of the most widely planted woody species. This genus has a complex taxonomy. One of its notable features is leaf dimorphism, i.e., juvenile and mature leaves differ in terms of shape, attachment and glaucousness (SALEHI et al., 2019).

In genomic research, the genus *Eucalyptus* has been a central focus, to which most of the literature is dedicated (GRATTAPAGLIA et al., 2012; MYBURG et al., 2014).

Over 1,098 species and subspecies of shrubs and trees belong to the genus *Eucalyptus*, which is native to Australia, Indonesia and neighboring islands, but has a vast number of recorded occurrences worldwide (FIGURE 1). In various countries, including Australia, Portugal, Japan, Colombia, the United States of America and Brazil, the economic importance of *Eucalyptus* is significant (GBIF, 2023)

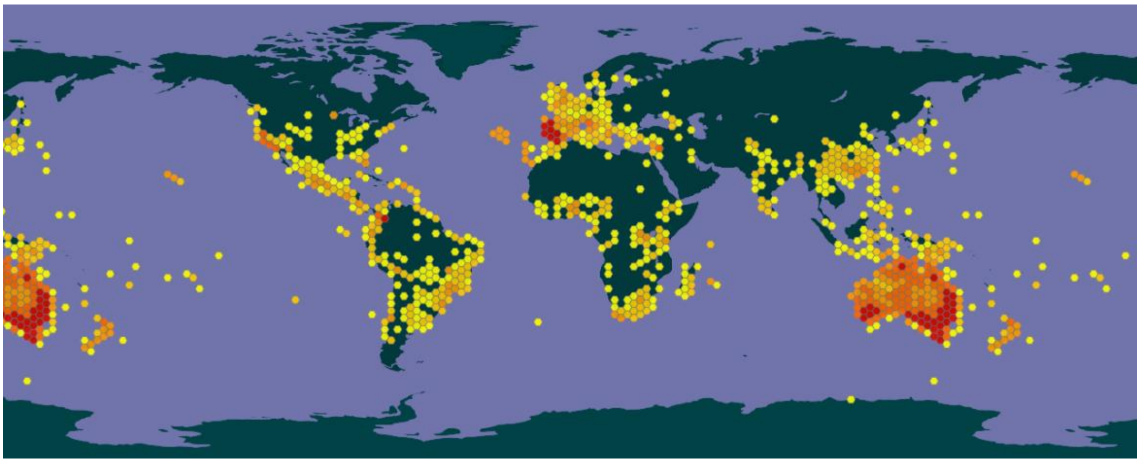


FIGURE 1 - Worldwide occurrence of *Eucalyptus*.

Economically, *Eucalyptus* is one of the highest-priced taxa of the order Myrtales, for being a renewable source of biomass for the furniture, plywood, fiber panel, pulp and paper industries, as well as for bioenergy (BROOKER, 2000; SANTAROSA; PENTEADO

JUNIOR; GOULART, 2014). *Eucalyptus* trees are known for their rapid growth, adaptability to diverse environmental conditions, drought tolerance and ability to thrive in impoverished or infertile soils (RAJ; JHARIYA; BARGALI, 2016; YU et al., 2016).

Due to this versatility, *Eucalyptus* can be cultivated in various regions. In southern Africa, South America, Asia and the Mediterranean region, it has been cultivated as an essential source of cellulose fiber (PAINE; STEINBAUER; LAWSON, 2011). With the growing demand for bioenergy and the need for sustainable alternatives, the species has the potential to become a significant renewable source for biofuel production (ROMANÍ et al., 2019).

Innovations in technological domains such as biotechnology, pharmaceuticals, food chemistry and basic materials have emerged, based on three *Eucalyptus* species (*E. grandis*, *E. camaldulensis* and *E. urophylla*), in particular on *E. grandis*. These innovations reinforce the indication of the species as a staple crop and as sustainable foundation of bioeconomy, while selection and breeding may still achieve higher yields and the introgression of desirable traits (CALAZANS et al., 2021).

Alternatively, *Eucalyptus* has been proposed as a source of microfibrillated cellulose (MFC), a component of hand sanitizers. During the COVID-19 pandemic, the increased demand for hand sanitizers led to market shortages. *Eucalyptus* cellulose is an affordable and environmentally friendly forest-based raw material.

In Brazil, *Eucalyptus* is one of the most important tree species due to its adaptability to different regions at similar latitudes as Australia and the versatile wood properties. However, despite the still expanding cultivation area, the cultivation of this tree species has been hampered by rising wood production costs (CASTRO et al., 2016).

Initially, the absence of pests and diseases facilitated the rapid expansion of *Eucalyptus* in Brazil. However, with the enlargement of the cultivation area, natural biodiversity declined, which resulted in pest epidemics (PAINE; STEINBAUER; LAWSON, 2011). Eventually, the insect communities gained a foothold in the cultivation areas (MANSFIELD, 2016).

Despite the significant ecological and economic importance of *Eucalyptus* species, the biological interactions between pests and these plants are still largely unknown (CARVALHO; CARVALHO, 2016). The globalization of trade and travel has contributed to a surge of invasive exotic insect pests, e.g., of the gall-forming hymenopterans. Over the past two decades, numerous gall-forming hymenoptera have become invasive pests of Eucalyptus, although a considerable number of these species had previously been identified as invasive pests. (DITTRICH-SCHRÖDER et al., 2020).

2.2 *Eucalyptus* key pests: impact in Brazil and worldwide

Pests represent constant challenges on *Eucalyptus* plantations. Insects can cause significant damage, affecting tree health and reducing productivity. Leaf-cutting ants, known for their classifications in *Atta* and *Acromyrmex*, play a detrimental role as pests on *Eucalyptus* plantations. Their destructive behavior consists of cutting fresh plant material, particularly the leaves, which are then transported to their nests.

Notable species among these ants are *Atta sexdens rubropilosa*, *A. laevigata*, *A. bisphaerica* and *A. capiguara*. Once the harvested foliage is inside the nest, the ants process it meticulously, creating a substrate for cultivation of fungi that are a vital food source for the ant colony.

The leaf-cutting ants have a major impact on plantations, as the defoliation they cause can significantly hinder plant development and disrupt the uniformity of the stand (REIS FILHO et al., 2021). In ongoing efforts to manage these pests, chemical control methods are still the most commonly applied and effective approach, relying mainly on the use of granular ant bait to contain insects and mitigate the damage they cause.

Another pest that attacks *Eucalyptus* is *Gonipterus* spp. (Coleoptera: Curculionidae). *Gonipterus platensis* (Marelli) and *G. scutellatus* Gyllynhal are defoliating beetles that damage leaves considerably (GONÇALVES et al., 2019; JORDAN et al., 2021; LOTTERING et al., 2019; SCHRÖDER et al., 2020; WILCKEN et al., 2008).

Phoracantha spp. (Coleoptera: Cerambycidae), specifically *P. semipunctata* (Fabricius) and *P. recurva* Newman, are longhorn borers, known for their ability to bore into *Eucalyptus* wood, where they cause severe economic damage (CORRÊA et al., 2020; MALUMPHY et al., 2020; MENDEL; PROTASOV, 2019; SEATON; MATUSICK; HARDY, 2020; ZHAO et al., 2023).

Phoracantha semipunctata is a highly destructive invasive exotic forest pest of worldwide occurrence. Primarily, it causes damage to trees at the adult stage and harms nearly all plant parts. In addition, its larvae develop on several major tissues of *Eucalyptus* trees (ZHAO et al., 2023). On the other hand, *P. recurva* is naturally found in Australia and the southern region of Papua New Guinea. This species has a high dispersal capacity and can survive for extended periods within stored logs (CORRÊA et al., 2020).

Several psyllid species, such as *Ctenarytaina eucalypti* Maskell (Hemiptera: Psyllidae), *C. spatulata* Taylor (Hemiptera: Psyllidae) and *Glycaspis brimblecombei* Moore (Hemiptera: Aphalaridae), are recognized as significant, globally distributed pests of

Eucalyptus (SILVA et al., 2022; SILVA; OLIVEIRA; MATA, 2023; SUMA et al., 2018). These insects damage the leaves, reducing the photosynthetic capacity of the trees (BRENNAN et al., 2001;

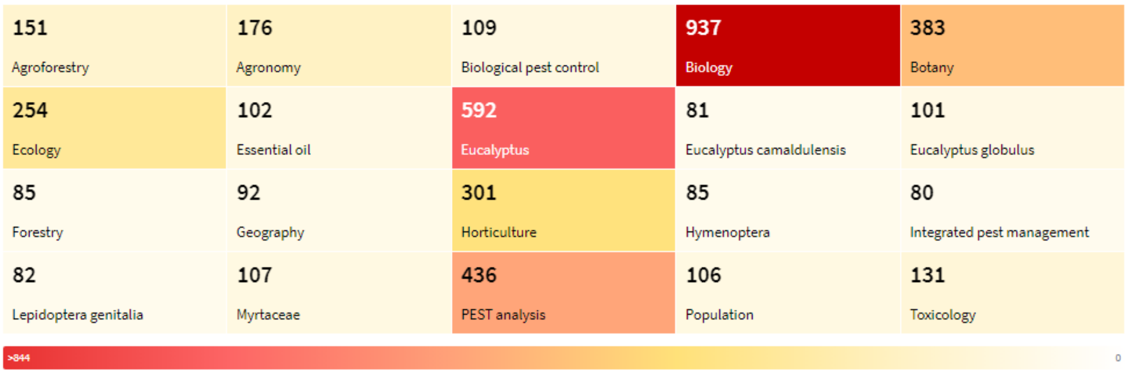
High *C. eucalypti* populations on primary shoots can lead to tissue drying, and consequently to changes in leaf coloration, deformation and shedding. Additionally, the blue gum psyllid can stimulate the production of new shoots, altering the growth pattern and plant vigor (KURYLO et al., 2010).

Infestation by *C. spatulata* typically results in deformed and smaller than normal leaves. Leaf curling and gaps between the veins can also be observed. The reduction in leaf size, along with the presence of honeydew, decreases the leaf surface area and consequently, the plant photosynthetic capacity (SANTANA; BURCKHARDT, 2007).

Glycaspis brimblecombei adults are grayish-orange to yellowish-green and have two pairs of wings. The nymphs are initially yellowish and develop dark-colored wing rudiments in the last stage. Females lay 45 - 700 eggs on open leaves, preferably on young leaves and branches. The embryonic period lasts 10 - 20 days and the hatched nymphs walk around on the leaves, to settle near leaf veins (MONTES; RAGA, 2005).

The bronze bug *Thaumastocoris peregrinus* D.L. Carpintero & Dellapé (Hemiptera: Thaumastocoridae) is a sap-sucking insect that affects *Eucalyptus* by causing damage to leaves and shoots (SOLIMAN et al., 2019).

Research focused on the pest-induced damage in *Eucalyptus* primarily addresses the understanding of the pest and host biology. Among the various insect orders, Hymenoptera stands out as a major contributor to the economic losses resulting from these pests. The implementation of an integrated pest management, based on biological pest control, is considered the most effective approach to deal with this issue. It is worth mentioning that to date, *E. camaldulensis* and *E. globulus* are the most extensively studied host species (FIGURE 2).



151 Agroforestry	176 Agronomy	109 Biological pest control	937 Biology	383 Botany
254 Ecology	102 Essential oil	592 Eucalyptus	81 Eucalyptus camaldulensis	101 Eucalyptus globulus
85 Forestry	92 Geography	301 Horticulture	85 Hymenoptera	80 Integrated pest management
82 Lepidoptera genitalia	107 Myrtaceae	436 PEST analysis	106 Population	131 Toxicology

FIGURE 2 - Top fields of studies about *Eucalyptus* pests.
Source: Created in Lens.org (2023).

In Brazil, several exotic insect pest species have been observed on *Eucalyptus*. These pests include the *Eucalyptus* weevils, *Gonipterus platensis* Marelli and *Gonipterus pulverulentus* Lea (Coleoptera: Curculionidae) (JORDAN et al., 2021; NANINI et al., 2022), as well as *Eucalyptus* longhorned borers, namely *P. semipunctata* and *P. recurva* (CORRÊA et al., 2020; SANTOS et al., 2007). The lemon-scented gum gall wasp *Epichrysocharis burwelli* Schauff and Garrison (Hymenoptera: Eulophidae) is another identified pest (PEREIRA, 2010), along with psyllids such as *Glycaspis brimblecombei* Moore (Hemiptera: Aphalaridae), QUEIROZ et al., 2010, 2018; SANTANA; BURCKHARDT, 2007). In addition, the bronze bug *Thaumastocoris peregrinus* Carpintero and Dellapé (Hemiptera: Thaumastocoridae) is another of various insect pests that affect commercial *Eucalyptus* forests in Brazil (SANTOS et al., 2016; SOLIMAN et al., 2012).

Leptocybe invasa, a gall-inducing wasp, forms galls on leaves and young branches, negatively impacting tree growth and overall yield (LITTLE; GARDNER, 2021; MPHAHLELE et al., 2021; PENG et al., 2021; SARMENTO et al., 2021). Several studies have documented the initial occurrences of *L. invasa* in various regions of the world (AQUINO; HERNÁNDEZ; ANDORNO, 2019; BARBOSA et al., 2018; PUSPASARI et al., 2020; VALENZUELA-ESCOBOZA et al., 2019).

To ensure tree health and productivity, effective management strategies in *Eucalyptus* plantations are imperative. Continuous monitoring, prevention and mitigation methods are necessary to minimize the impact of these pests and maintain sustainable forestry practices.

2.3 *Leptocybe invasa*: a devastating invasive pest threatening *Eucalyptus* plantations

Leptocybe invasa Fisher & La Salle, 2004 primarily attacks young plantations and has a devastating impact on nursery seedlings (GALLFORMS, 2023; MENDEL et al., 2004a; MENDEL; PROTASOV, 2019; YOUSUF, 2019). The galls induced by *L. invasa* are found on the petioles, leaf midribs and young branches of *E. camaldulensis, E. dunni*, *E. grandis*, *E. globulus*, *E. longistrata*, *E. major, E. propinqua*, *E. robusta*, *E. rudis*, *E. tereticornis,* and *E. viminalis* (DHAHRI et al., 2010; ESKIVISKI et al., 2018; GALLFORMS, 2023; MHOSWA et al., 2020).

With a body length of approximately 1-1.5 mm, *L. invasa* is a small wasp reportedly native to Australia (KENIS et al., 2019). The reproduction mode (thelytokous and sexual) is advantageous for rapid colonization and for outbreaks of this species (ZHENG et al., 2014, 2018).

The female *L. invasa* lays eggs on plant tissues, inducing gall formation on the leaf midribs, petioles and young branches (NUGNES et al., 2016). This results in leaf curling and premature aging (FIGURE 3). Heavy egg infestation can cause the death of young shoots, while severe wasp attacks lead to leaf drop, stunted growth and can ultimately weaken the tree, resulting in significant losses in *Eucalyptus* plantations (FERNANDES et al., 2014; HUANG et al., 2018; MENDEL et al., 2004a; YOUSUF, 2019; ZHENG et al., 2014).

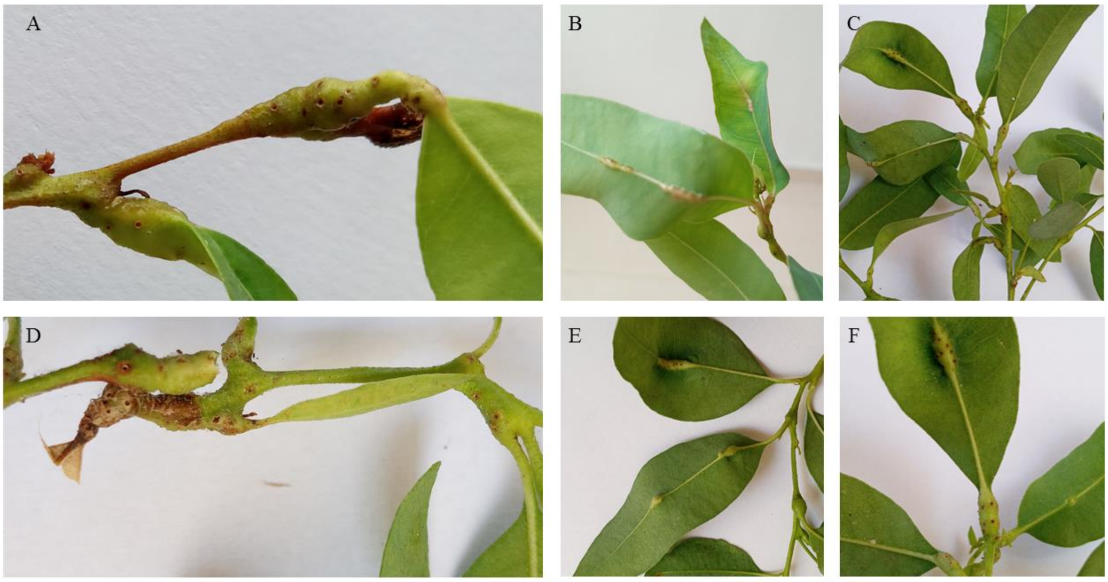


FIGURE 3 - *Leptocybe invasa* galls on *Eucalyptus*: galls with exit holes on branches and leaf petioles: A- and D galls on petioles; B, C, E and F - galls on leaves and petioles.
Source: Author’s own pictures (2023).

The mean length of a gall with a single wasp is approximately 2.1 mm and rapidly growing trees can have over 50 galls per leaf (DANTAS, 2019; MENDEL et al., 2004a). The female wasps lay about 100 eggs, preferably beside the petiole, i.e., the leaf stalk (DHAHRI et al., 2010). As the larvae develop, galls grow and the affected leaves turn glossy pink (FIGURE 3 – E and F). Over time, the glossiness of the petiole diminishes and the gall color changes from pink to red (FIGURE 3- A). When the wasps emerge, the galls on the petiole turn light brown and the galls on the stems reddish-brown (FIGURE 3 – D). After emergence, the adults leave round exit holes in the galls.

The infestation of *L. invasa* disrupts the arrangement of organic solutes within the foliage, interfering directly with photosynthesis. Consequently, the forestry production of *Eucalyptus* is significantly impacted (DANTAS et al., 2021).

Understanding the characteristics of *L. invasa* is important to develop effective management strategies. This knowledge can contribute to define specific control measures and minimize the damage caused by *L. invasa* infestations (AHMED et al., 2017).

The detrimental effects caused by *L. invasa* call for effective management strategies to mitigate the damage and ensure yield stability and sustainability of plantations.

2.4 Distribution of *Leptocybe invasa* in Brazil and worldwide

Leptocybe invasa, first described in 2004 (MENDEL et al., 2004b), is believed to be native to Australia or the native range of its host plants, including *Eucalyptus* species in Australia, New Guinea, Indonesia and Philippines. However, it has only been detected in Queensland and New South Wales where it is known for its high damage capacity and rapid reproduction (NUGNES, 2022). The genus *Leptocybe* and its type species *L. invasa* are relatively recent advances in scientific knowledge. The first recorded occurrence dates back to 2000 in Israel. By 2018, *L. invasa* infestations had been reported in 45 countries across five zoogeographic regions (LE et al., 2018).

The expansion of this invasive pest has been facilitated by international trade and the movement of infested plant material. The economic impact of *L. invasa* is significant, since it reduces timber quality and yield and raises production costs due to the need to invest in pest management (FERNANDES et al., 2014; NUGNES et al., 2015, 2016). Over the past two decades, *L. invasa* has spread globally (FIGURE 4), invading all continents where *Eucalyptus* was introduced, including Asia, Africa, Europe, South America and North America (ZHENG et al., 2014).

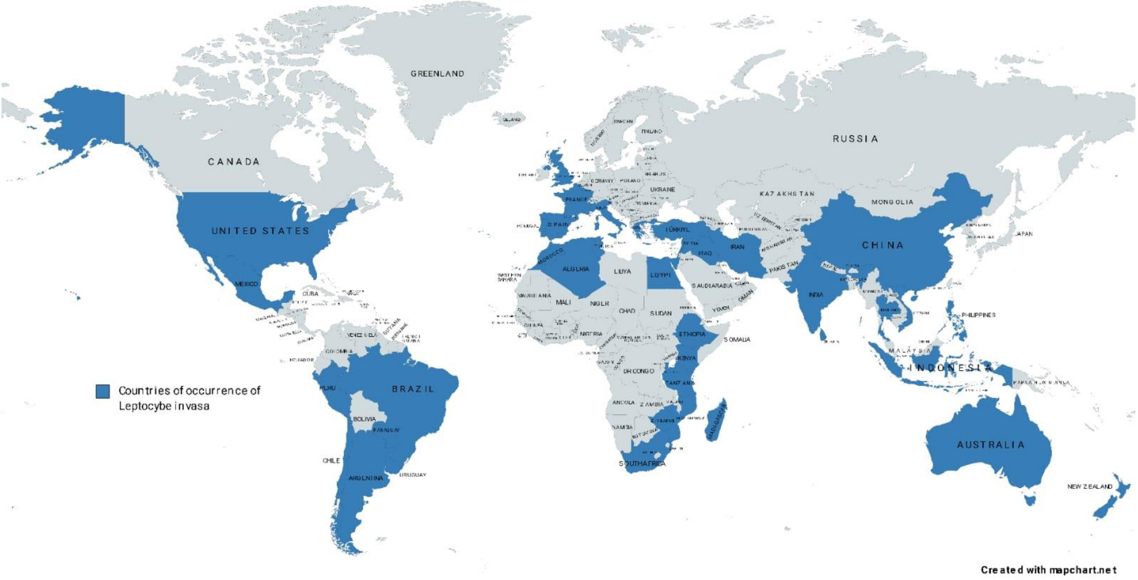


FIGURE 4 - Geographic distribution of the gall wasp (*Leptocybe invasa*) in 2023.
Source: Author’s own map (2023).

Studies have reported the occurrence of *L. invasa* in various countries, including Algeria, Australia, China, France, India, Indonesia, Iran, Israel, Italy, Jordan, Kenya, Mexico, Morocco, South Africa, Spain, Syria, Tanzania, Thailand, Turkey, Uganda, Uruguay, USA and Vietnam BARBOSA et al., 2018; DITTRICH-SCHRÖDER et al., 2014; GEVERS et al., 2021; HUANG et al., 2018; JORGE et al., 2016; NDLELA et al., 2018; PROTASOV et al., 2008; RAMANAGOUDA; VASTRAD, 2015; SOUZA TAVARES et al., 2023; ZHANG et al., 2021; ZHENG et al., 2016).

In Brazil, the pest was first reported in the state of São Paulo, in 2008. Since then, it has spread to several *Eucalyptus*-growing regions of the country. The geographic distribution of the gall wasp is a great concern in Brazil, due to its significant impact. The gall wasp has been reported in the states of *Eucalyptus* cultivation, i.e., in 17 of all 26 states of Brazil (FIGURE 5), namely in: Alagoas (AL), Bahia (BA), Espírito Santo (ES), Goiás (GO), Maranhão (MA), Minas Gerais (MG), Mato Grosso (MT), Mato Grosso do Sul (MS), Pará (PA), Piauí (PI), Paraná (PR), Pernambuco (PE), Rio Grande do Sul (RS), Santa Catarina (SC), São Paulo (SP), Sergipe (SE), and Tocantins (TO) (BARBOSA et al., 2018; FERNANDES et al., 2014; GARLET et al., 2013; PEREIRA et al., 2014).

FIGURE 5 - Geographic distribution of the gall wasp (*Leptocybe invasa*) in Brazil in 2023.
Source Author’s own map (2023).

The expanding geographic distribution of *L. invasa* in Brazil can be attributed to the extensive *Eucalyptus* plantations in these states, which creates a favorable environment for pest establishment and proliferation (LEMES; ZANUNCIO, 2021).

Multiple factors influence *L. invasa* distribution, for example climatic conditions and host availability. Climate, in particular, plays a significant role in determining the pest establishment and spread to new regions, as the *L. invasa* life cycle requires specific temperature and humidity conditions (YANG et al., 2015).

2.5 Characteristics and life cycle of *Leptocybe invasa*

Adult *L. invasa* wasps are characterized by their small size (body length 1.1-1.4 mm) (FERNANDES et al., 2014). Antennae and legs of *L. invasa* are brown, while the basal joint of the antennae is yellowish. The scape of the antennae is longer than the pedicel and has a broadened middle section. The flagellum consists of six segments separated by four annelli. The basal segments form the funiculum and the three apical segments the antennal mass (JORGE et al., 2016).

The species is known to reproduce by thelytokous parthenogenesis, where only females are present and can give rise to new females without the need for males. These female wasps (FIGURE 6) have a dark brown body with a metallic blue-green gloss, particularly on head and thorax, while the antennae and legs are brown, and yellowish at the basal joint (WILCKEN et al., 2011).

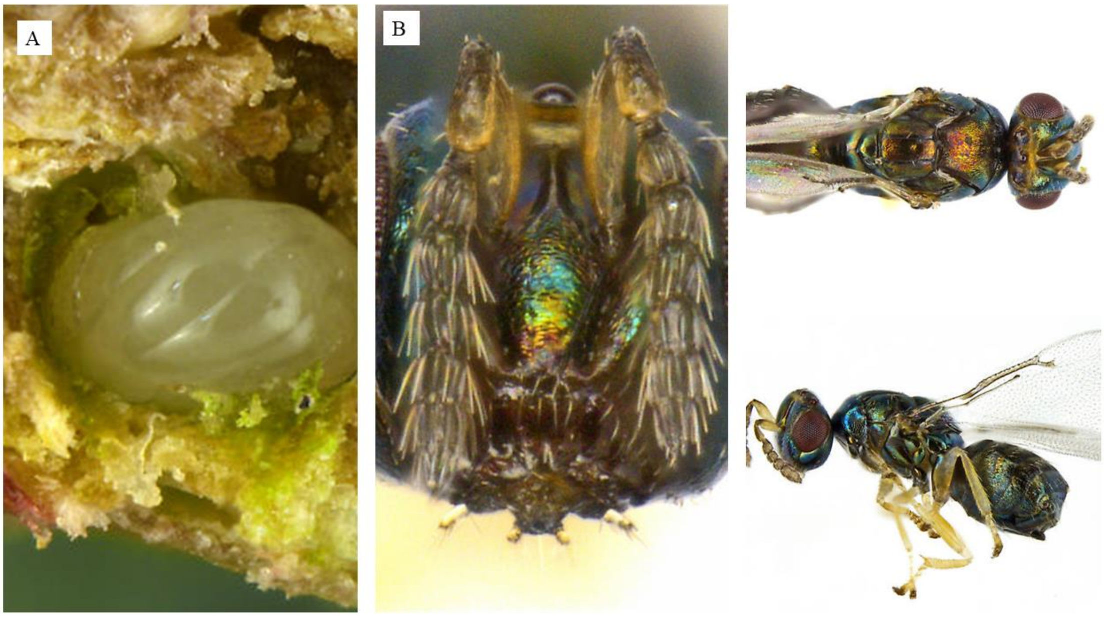


FIGURE 6 - *Leptocybe invasa*. A – Larva in the gall; B - Female 1.7 mm.
Source: BugGuide (2023).

The life cycle of *L. invasa* varies according to the geographical region. The duration of the biological cycle, from oviposition to adult emergence, lasts approximately 130 days (WILCKEN et al., 2011). In Israel, a cycle of 136 days has been reported, while in the region of Botucatu, Brazil, the wasps have a shorter cycle (80 days), similar to the 91-day cycle observed in South Africa. However, in comparison to Thailand, where a 45-day cycle has been observed, the life cycle in Brazil generally tends to be longer (DITTRICH-SCHRÖDER et al., 2014; SANGTONGPRAOW; CHARERNSOM; SIRIPATANADILOK, 2011).

Temperature significantly influences the developmental period and survival rate of *L. invasa*. In a controlled temperature range of 20 to 32°C, the developmental period from egg to adult is shortest at 29°C and longest at 20°C. The highest number of eggs per female and highest survival rate of immatures were recorded at 29°C, with a more pronounced temperature effect on females than males (ZHU et al., 2015).

Understanding the life cycle of *L. invasa* is essential for the development of integrated pest management strategies. Studies have shown the efficacy of insecticide application during specific phases of the life cycle, while also suggesting the possibility of biological control during other phases (LUNA-CRUZ et al., 2020).

The life cycle of *L. invasa* exhibits remarkable variations across different regions, highlighting the impact of local environmental conditions. Research findings from diverse geographical locations provide insights into the duration and characteristics of the developmental stages.

2.6 Integrated pest management for *Leptocybe invasa* control

Integrated Pest Management (IPM) is a holistic approach to combat pests, with a combination of multiple complementary tactics, including monitoring, cultural practices, physical and mechanical measures, host plant resistance, biological control and targeted pesticide use (GREEN; STENBERG; LANKINEN, 2020; MOUDEN et al., 2017).

Prevention is a key aspect of pest management, with a view to reducing the likelihood of pest populations reaching levels that require control measures. This is achieved by implementing measures that create an unfavorable environment for pests and reduce plant vulnerability to pest attacks (ROSSI et al., 2019).

Within the IPM framework, various strategies are employed to control *L. invasa* (FIGURE 7). For example, biological control agents are introduced to suppress the *L. invasa* population (BARBOSA et al., 2018; GEVERS et al., 2021; HURLEY et al., 2017; KIM et al., 2008; MASSON et al., 2017; MENDEL et al., 2017; NDLELA et al., 2018; SANGTONGPRAOW; CHARERNSOM, 2013; SOUZA TAVARES et al., 2023).

Cultural control practices, e.g., the pruning of infected branches and removal of affected plant material, contribute to reduce the severity of the pest attack. It is worth remembering, however, that short rotations of trees planted at the same sites can lead to an accumulation of populations of soil-borne insects and microbial pathogens (ODHIAMBO; WANJALA; KHAEMBA, 2013; ROY; MITRA, 2013; ZHENG et al., 2016).

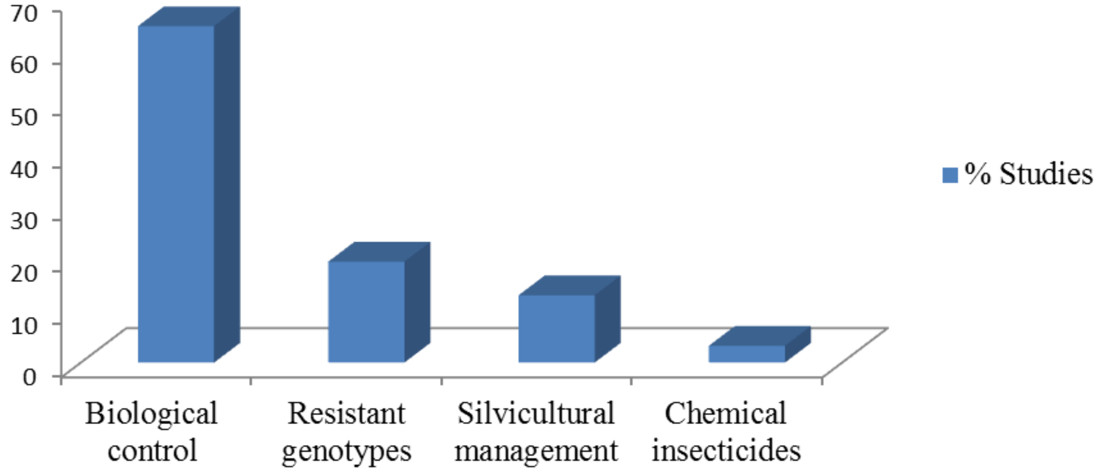


FIGURE 7 - Strategies used around the world for an integrated pest management of *Leptocybe invasa*, based on Web of Science and Scopus studies.
Source: Author's own diagram (2023).

Physical control methods, such as the use of barriers and exclusion nets, are effective in preventing adult wasps from laying eggs on susceptible plant parts (WINGFIELD et al., 2020). Chemical control measures should be employed cautiously and as a last resort, in view of their potential impact on non-target organisms and the environment (AREGOWDA; PRABHU; PATIL, 2010; CHEN; JIN; TU, 2015; JAVAREGOWDA; PRABHU, 2010; LUNA-CRUZ et al., 2020).

One of the strategies consists of a range of biological control agents, such as parasitoid wasps, predators and entomopathogenic fungi that can diminish pest populations. Additionally, cultural practices such as meticulous sanitation and the removal of infested plant parts are key to diminish pest pressure.

Although chemical control methods require double caution, due to possible environmental consequences, they are still an element of the integrated approach. Insecticides are carefully applied during specific life stages of the pest to minimize harm to non-target organisms.

Moreover, host plant resistance is pursued by the identification and cultivation of *Eucalyptus* genotypes presumed to have inherent resistance to gall wasps. This approach is notable for its efficacy, since the relevance of plant resistance for *L. invasa* control will not decline over the course of time.

2.6.1 Mechanisms of plant resistance against insect pests

Plant resistance to insects primarily relies on inherited genetic traits related to reduced plant vulnerability, compared to susceptible counterparts under equivalent conditions (MOOKIAH et al., 2021). Plant- and insect-related factors can influence resistance expression, including plant age, attacked plant part, prior damage occurrence, insect developmental stadium and pest species, race or biotype and population size (MORAIS; PINHEIRO, 2012).

There are three functional categories of insect resistance: non-preference, antibiosis and tolerance resistance. These categories typically do not occur separately, since plants can exploit various combinations of the three (GALLUN, 1972; WISEMAN, 1985).

Non-preference resistance, also known as antixenosis, means that plants are less accepted by insects, for feeding, oviposition and shelter, than other plants under the same conditions. Non-preference measures are disincentives, based on chemical and physical plant defenses that prevent insect colonization and feeding (GALLUN, 1972).

Tolerance is based solely on plant traits and the plant capacity to endure damage caused by insect feeding. A plant is considered tolerant if the pest damage level is lower than that of others at the same insect infestation level, without affecting behavior or biology of the insect. A tolerant plant withstands insect damage by tissue regeneration, new shoot or tiller emergence or other mechanisms that prevent production reductions (WISEMAN, 1985).

Antibiosis is given when insects feed on plants that adversely affect insect development. These effects may consist of insect mortality (or survival) at different developmental stadiums, size and weight reduction of individuals, alteration of sex ratios, and changes in lifespan. Antibiotic factors comprise toxins, growth inhibitors, reduced nutrient levels and high concentrations of indigestible plant components (SMITH, 2005).

Host plants of a same species can have distinct responses in gall formation, according to the genotype (ISAIAS et al., 2015). In the context of *L. invasa* infestations, an intriguing aspect of the interaction lies in the diverse tolerance levels different *Eucalyptus* genotypes can have when attacked by *L. invasa*.

To develop an integrated management program against gall wasp infestation, genetic resistance is the ideal method as it does not directly increase production costs and is risk-free (VIDA et al., 2004). Plant genetic resistance consists of using immune or tolerant plant varieties to prevent or control diseases. In Uganda, farmers have reported achieving higher yields by planting recommended resistant *Eucalyptus* hybrids. However, they were unable to specify which hybrids were resistant and which ones were susceptible to the insect (NYEKO; MUTITU; DAY, 2007).

Studies on the reasons for *L. invasa* resistance and susceptibility of *Eucalyptus* germplasms are scarce in the literature (NYEKO; MUTITU; DAY, 2009). However, as is generally known, genotype resistance can be influenced by external factors such as the environment, nutrients, microbes and compounds used in plant treatments.

Some species are susceptible to gall formation and should be avoided in nurseries: *E. camaldulensis*, *E. saligna*, *E. botryoides*, *E. bridgesiana*, *E. cinerea*, *E. globulus*, *E. grandis*, *E. urophylla*, *E. gunnii*, E. *nicholii*, *E. pulverulenta*, *E. robusta*, *E. rudis*, *E. tereticornis*, and *E. viminalis* (FAO, 2012; MENDEL et al., 2004). On the other hand, some species and their hybrids are resistant to gall formation even after oviposition occurs (DANTAS, 2019).

The use of pathogen-resistant genotypes represents the best alternative for pest control. Traditionally, trees with desired properties were developed in long-term breeding programs. However recently, genomic and transcriptomic studies have emerged as promising tools, applied in breeding programs by marker-assisted selection (NASCIMENTO et al., 2017).

The use of resistant varieties from genetic studies can be identified in various countries, as indicated by patent deposit data (FIGURE 8).

FIGURE 8 - Distribution of patent applicants for *Leptocybe invasa* pesticides per country.
Source: LENS, 2021.

With regard to the countries of origin of the main insecticide *or* pesticide patents for *L. invasa*, China is leading (11 publications), followed by South Africa (5), the United Kingdom

(4), Brazil (3), India, Tanzania and Uganda (2 articles each), and 12 other countries with one publication each.

2.7 Forest Stewardship Council (FSC) forest certification and integrated forest pest management

The Forest Stewardship Council (FSC) is a renowned non-governmental organization working on the sustainable management of forest resources and that considers social, environmental and economic factors. One of the key standards of FSC certifications is the emphasis on integrated pest management (IPM) practices, which play a crucial role in ensuring sustainable forest management and minimizing the use of chemical pesticides.

Forestry enterprises with a concern for environmentally sound, socially beneficial and economically viable standards adhere to a "sound forest management" by having their forest products certified by FSC. An additional goal is to assure the traceability of products derived from these sources. Forest certification serves as a strategic instrument of aligning forest management with the baselines of sustainable development

In Brazil, this certification framework is provided by the Brazilian Forest Certification Program (CERFLOR). On a global scale, the Forest Stewardship Council (FSC) and the Program for the Endorsement of Forest Certification (PEFC) are internationally renowned systems. Established in 1993, the FSC, as a groundbreaking non-governmental organization composed of stakeholders, is dedicated to ensuring the social, environmental and economic sustainability of forest resources (RUA et al., 2009).

A priority concern of FSC is the use of an integrated pest management (IPM), which should diminish or obviate chemical pesticide applications in management units (MUs), with a view to mitigating risks to human and environmental well-being, while simultaneously maintaining the economic viability of the forestry practices (FSC, 2019a).

A FSC certification seal on products obliges the forest enterprises to adhere to the 10 principles and 56 criteria established by the institution. By incorporating policies of social responsibility, these companies adopt practices that are not only socially and environmentally conscious but may also be a doorway to new markets. An inherent part of the "Principles & Criteria" is the FSC Pesticide Policy that demands the adoption of integrated pest management and silviculture systems, which curtail or eliminate the use of chemical pesticides (FSC, 2019a).

It must be emphasized that the FSC acknowledges the possible need of applying chemical pesticides under specific circumstances. Based on a comprehensive evaluation of available pest management strategies and practices, the FSC clearly states that chemical pesticides may represent the sole feasible method for pest control in certain instances (FSC, 2019b).

To achieve FSC certification and effectively implement IPM practices for *Leptocybe invasa* control, forest enterprises should:

- carry out detailed assessments of the severity and distribution of pest infestations; study and implement non-chemical pest control methods, prioritizing the biological, mechanical and cultural control; monitor and evaluate the effectiveness of IPM strategies, making adjustments as needed; keep detailed records of pest management activities and their outcomes to demonstrate compliance with FSC standards; cooperate with experts, researchers and other instructors to stay updated on the latest IPM techniques and advancements.

While the FSC encourages a minimal use of chemical pesticides, the organization acknowledges that in some cases, after careful consideration of available alternatives, their use may be unavoidable. Effective IPM practices are crucial for addressing pests such as *L. invasa* without losing the ecological balance of forest ecosystems and while meeting the FSC certification standards.

2.8 Genotype characterization: transposable elements (TE) and inter simple sequence repeat (ISSR) markers as tools to detect resistance

Distinguishing genotypes accurately with genetic markers is essential for the identification of pest-resistant plants and the development of resistant varieties, to make the implementation of highly effective pest management strategies possible.

Transposable Elements (TEs) are DNA sequences capable of relocating within a genome (OCHMANN; IVICS, 2021). They are prevalent across various organisms and play a role in genetic diversity and evolution. Transposable Element markers have been extensively used for genotype discrimination due to their genome-wide distribution. These markers are classified primarily into Class I and Class II, based on their transposition mechanisms (LISCH; BENNETZEN, 2011; ZHANG et al., 2020).

Class I elements, also known as retrotransposons, are characterized by their ability to transpose via RNA intermediate. These elements move within the genome by means of a "copy and paste" mechanism. The process involves the transcription of the retrotransposon DNA into RNA by a host RNA polymerase. The RNA is then reverse transcribed into DNA by the enzyme reverse transcriptase. The resulting DNA molecule, known as cDNA, is subsequently integrated into a new genomic location by an integrase enzyme. This process triggers the amplification and proliferation of retrotransposons throughout the genome (FESCHOTTE; JIANG; WESSLER, 2002; ZHANG et al., 2020).

Class II elements, also called DNA transposons, move within the genome through a "cut and paste" mechanism. These elements encode a transposase enzyme that recognizes specific DNA sequences and catalyzes their excision from one genomic location and subsequent insertion into another. Unlike for retrotransposons, no RNA intermediate is involved in the transposition process of DNA transposons (ALZOHAIRY et al., 2014) (FIGURE 9).

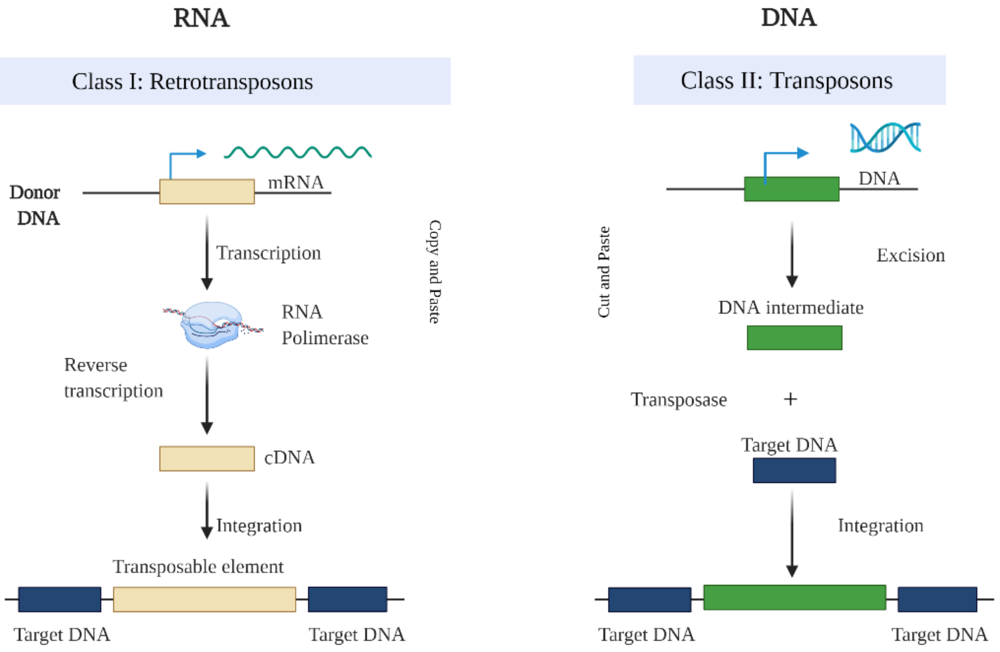


FIGURE 9 - Classification of transposable elements.
Source: Author’s own diagram (2023).

The genomes of most plant species are strongly influenced by transposable elements (TEs), which play a significant role in driving genome evolution. In recent years, environmental stimuli have been found to activate specific TE families, leading to new insertions. This dynamic process can result in altered gene expression and phenotypes, providing plants with powerful mechanisms to adapt to changing environmental conditions (DUBIN; MITTELSTEN SCHEID; BECKER, 2018).

Eucalyptus. grandis was the first species of the genus subjected to genome sequencing, which detected that nearly half of the genome consists of retrotransposons (KALENDAR et al., 2011; MARCON et al., 2015). These data are important to understand the dynamics of transposable elements in the *Eucalyptus* genome and transcriptome. However, studies on transposable elements in this genus are still scarce.

Research on transposable elements has primarily addressed three key areas. Firstly, the evolution and genomic associations of transposable elements have been a study focus, often centered on the model species *Arabidopsis thaliana*. Secondly, phylogenetic analysis have been performed to identify and explore TEs across different species. Lastly, the role of transposable elements in gene conservation and the identification of genes of interest have been a key focus of investigation. These three lines of research are represented by the major clusters in Figure 10.

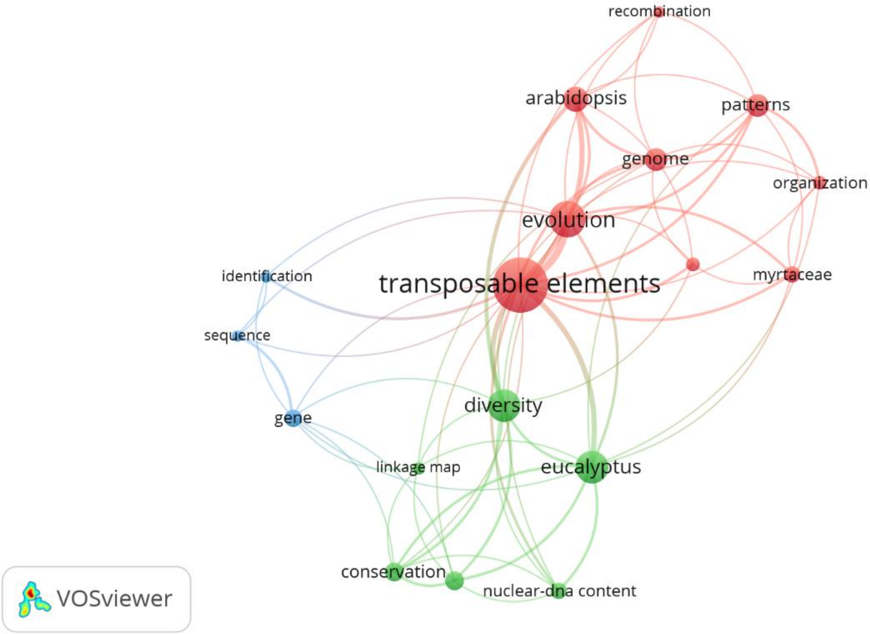


FIGURE 10 - Scientific review about network of interactions between *Eucalyptus* and transposons, based on Web of Science and Scopus studies.
Source: Author’s own diagram (2023).

Retrotransposon-based molecular marker studies with LTR regions have gained attention. Research based on this system is effective and is being applied to a range of cereals, grasses, as well as species such as cashew, coconut, tomato, pepper, legumes, fungi, birds and insects. Applications range from investigations of retrotransposon activation, their mobility for biodiversity studies and genome evolution to gene mapping and genetic distance estimation, as stated by Kalendar et al. (2011).

Inter Simple Sequence Repeat (ISSR) markers use Simple Sequence Repeats (SSR) repeat-anchored primers to amplify DNA segments located between two identical SSR repeat regions in opposite directions (REDDY; ALI; REDDY, 2002). Inter Simple Sequence Repeat markers are multi-locus molecular markers widely employed in genetic diversity analysis for region amplification between microsatellite ends. Their ability to generate multi-locus data and high genetic variability make them suitable for genetic diversity analysis (DARYONO et al., 2019). Moreover, ISSR markers are advantageous, as they do not require prior knowledge about the target sequences in the genome (SAMAL et al., 2012).

Inter Simple Sequence Repeat markers have proved to be efficient in discriminating genotypes across multiple crops. They have been used successfully to differentiate genotypes in *Sesamum indicum* L. (SHARMA; KUMAR; MATHUR, 2009), examine intraspecific relationships among different genotypes of *Mangifera indica* L. (SAMAL et al., 2012) and screen *Bt* cotton genotypes (ASHRAF et al., 2016).

2.9 Bioinformatics to detect genes of interest for *Leptocybe invasa* resistance

Bioinformatics is a hybrid science that integrates biological data with data storage, distribution and analysis techniques. It relies on biological databases to support the analysis of large-scale genetic datasets, making the identification of genes and pathways associated with resistance traits in the context of *Leptocybe invasa* possible (OCHMANN; IVICS, 2021).

Bioinformatics tools provide functional annotation of genes, linking them to specific biological processes, molecular functions and cellular components. They serve to deepen the understanding of underlying molecular mechanisms and the identification of key resistance-related genes (OCHMANN; IVICS, 2021).

Moreover, knowledge of genomic sequences extracted from deposited sequences for comprehensive analyses can enhance comparative studies among related species (KARAKÜLAH; PAVLOPOULOU, 2018). Transcriptomic approaches have been applied to characterize changes in global gene expression in response to pathogens, including *L. invasa* (SANA et al., 2010).

In silico analyses are computational methods and simulations used to study biological processes and systems. Virtual or simulated observations can be validated by comparing them to experimental data. In the context of *L. invasa* resistance, *in silico* analysis confirms observations that reflect plant metabolic and genetic responses to gall-wasp, which is useful in the identification of potential resistance mechanisms (LI et al., 2017; MAGED, 2016; PINSUPA et al., 2023).

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4. MANUSCRIPT 1

INNOVATIVE APPROACHES TO *Eucalyptus* RESISTANCE: A META-ANALYSIS OF GENE ASSOCIATIONS FOR *Leptocybe invasa* CONTROL

ABSTRACT

The objective of this paper was to explore in detail the current state of knowledge on *Leptocybe invasa* in *Eucalyptus* and identify the genes and metabolic pathways associated with plant-insect interactions, with a view to controlling this pest more effectively. In a comprehensive investigation across scientific articles, gene and protein sequences and patents, information on pest resistance in *Eucalyptus* spp was compiled. A literature survey was carried out in March 2023, using the keywords ("*Leptocybe invasa*" OR "*Eucalyptus* gall wasp"). Articles were retrieved from the scientific databases Scopus and Web of Science and analyzed using the Bibliometrix R-package. Genes annotated for *Eucalyptus grandis* were obtained from Phytozome v7.0. Gene pathways were identified using the Plant Reactome Gramene Pathways database. Patents were sought using the Orbit Intelligence patent research software, considering depositor, inventor, citation, international classification and geographical and temporal distribution. A total of 234 documents were included in the analysis, of which 116 were retrieved from the Web of Science and 118 from Scopus. Plant Reactome Gramene Pathways and functional analyses detected a pathway related to the gene Eucgr.B02310, associated with terpene compound synthesis. The studies demonstrated that α-phellandrene, linalool, α-terpinyl acetate and viridiflorol were produced by all tested *Eucalyptus* genotypes with *L. invasa* resistance. Most patents related to *L. invasa* control were related to pesticide development. Notably, an invention was launched that attracts and traps *L. invasa* on a viscous plate by a volatile component. In the scientific review, the host-insect relationship, management strategies and physiology and origin of *L. invasa* were represented by distinct research clusters.

Keywords: Patents; pest tolerance; protein.

4.1. Introduction

The *Eucalyptus* industry plays a vital role in meeting the worldwide demand for timber, pulp and renewable energy sources. The reasons behind the growing need are diverse and related to different factors, e.g., the rising wood consumption in thriving economies such as India, Brazil and China, increasing world population and as alternative to illegal logging. In addition, the conversion of native to agricultural land in tropical regions is causing a decline in naturally forested areas, increasing the dependence on plantations. As a result, forest management has become a global activity, characterized by changing ownership patterns (McEwan et al. 2020).

Since the 2000s, *Leptocybe invasa* has come to be a major threat to *Eucalyptus* spp. trees. This tiny gall-inducing wasp targets primarily *Eucalyptus* seedlings in nurseries and young stands. The infestation caused by *Leptocybe invasa* induces gall formation, stunted growth and a deformed tree structure. These detrimental effects have a significant impact on timber quality, reduce the productivity and result in economic losses for *Eucalyptus* growers worldwide (Oulmi et al. 2020).

Commonly known as the *Eucalyptus* gall wasp (Huang et al. 2022), *Leptocybe invasa* Fisher & La Salle 2004 (Hymenoptera: Eulophidae) is a notorious pest that causes substantial damage to *Eucalyptus* trees. The existing control methods against *L. invasa* have not been effective, which increases the challenges associated with its control. Although natural resistance of certain *Eucalyptus* varieties to gall wasp infestation has been observed, the genetic and molecular basis of this resistance remains unexplored.

The limited understanding of the biology, genetics and interactions between *L. invasa* and *Eucalyptus* plants has hindered the development of effective management strategies (Dittrich-Schröder et al. 2020). Information that can contribute to establish appropriate control strategies and reduce productivity losses in the forestry sector must urgently be collected, in view of the ecological and economic importance of *Eucalyptus* (Carvalho and Carvalho 2016; Oliveira et al. 2022).

The resistance level of *Eucalyptus* trees against gall wasp is closely related with a sustainable management of *Eucalyptus* plantations. Understanding the mechanisms underlying resistance and identifying key genes, protein sequences and pathways involved in this characteristic are essential steps towards developing effective strategies to combat gall wasp infestation.

By means of a scientific review, the purpose of this study was to explore innovative technologies and potential genes and metabolites linked to *Eucalyptus* resistance against *L. invasa* attack.

4.2. Material and Methods

The methodology involved the search for and review of recent scientific articles related to *L. invasa* tolerance in *Eucalyptus*. Secondly, potential factors that can influence this tolerance were sought among genetic and chemical compounds. In addition, technological patents related to the topic were reviewed.

4.2.1. Scientific mapping

A thorough literature search was conducted in March 2023, using the keywords "*Leptocybe invasa*" OR "*Eucalyptus* gall wasp". Articles in the scientific databases Scopus (http://www.scopus.com) and Web of Science (http://www.webofknowledge.com) were screened. The metadata of scientific publications resulting from each search term in both databases were exported in BibTeX format, excluding duplicate files. These datasets were combined and examined by meta-analysis evaluation techniques, e.g., productivity measures (number of articles per academic year, number of articles per author) and impact metrics (total number of citations, number of citations within a given period, number of citations per author). The Bibliometrix R-package (Aria and Cuccurullo 2017) was used for these analyses.

The existing academic knowledge was analyzed based on conceptual aspects, by means of co-word analysis, using VOSviewer software, version 1.6.16 (VOSviewer 2023). Articles were selected based on the evaluation of title, abstract and keywords. By the PRISMA method (Page et al. 2021), articles were selected according to predetermined inclusion and exclusion criteria (Figure 1). All articles identified in the refined search were scrutinized.

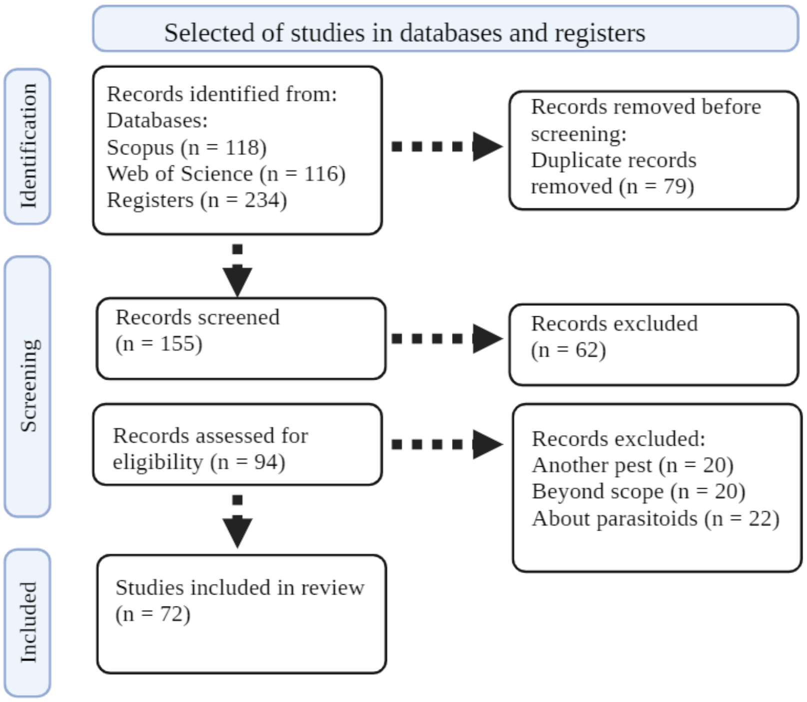


Figure 1 - Flowchart of scientific mapping based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

The website <https://www.gbif.org/> was used to verify the occurrence and taxonomic correspondence of the parasitoids used in the biological control of *Leptocybe invasa*.

4.2.2. Data mining of genes and secondary compounds

Genes annotated for *Eucalyptus grandis* were downloaded from Phytozome v7.0 (http://phytozome.jgi.doe.gov). The data included gene ontology (GO) terms, which provide standardized descriptions of gene functions and biological processes. By accessing this annotation information, the functional characteristics of specific genes in *Eucalyptus* could be analyzed and interpreted, contributing to a better understanding of their roles and potential functions in the plant. The nucleic acid sequences were searched in FASTA format among the collection of all publicly available DNA sequences in the Genetic Sequence Database-GenBank (http://www.ncbi.nlm.nih.gov/genbank). Molecular structures of secondary compounds were searched in the database ChEBI (Chemical Entities of Biological Interest).

To identify pathways, the Plant Reactome Gramene Pathways database (https://plantreactome.gramene.org/index.php?lang=en) was used. This database provides comprehensive information on plant metabolic and signaling pathways, making analysis and interpretation of gene functions in the context of biological pathways possible (Plant Reactome 2023), by the following methodology:

1. Gene Search: The gene identifier or sequence was entered into the search bar on the website. The search was performed and relevant results were recorded.
2. Pathway Analysis: The pathways associated with the gene of interest were explored in the Plant Reactome Gramene Pathways database. Pathway names, descriptions and other relevant information were reviewed to determine the potential involvement of the gene in specific pathways.

4.2.3. Patent prospecting

Patents were analyzed in Orbit Intelligence patent research software, for applicant, inventor, citation, international classification and geographical and temporal distribution (Questel Orbit® 2021). Documents with one of the terms “*Leptocybe invasa*” OR “*Eucalyptus* gall wasp” in the title, summary and description were taken into consideration. No time period was defined.

4.3. Results and discussion

4.3.1. Scientific mapping

A total of 234 documents were included in the analysis, 116 of which were retrieved from the Web of Science and 118 from Scopus. After data processing and removal of duplicates (n = 79), 155 documents, consisting of articles published between 2004 and 2023, were maintained in the study. In a thorough review of titles and abstracts, the eligibility criteria were applied. This resulted in the exclusion of 20 articles related to another pest, 20 articles beyond the scope of this research and 22 studies on parasitoids unrelated to *L. invasa*. Based on this refinement, (n = 94) articles were maintained for full-text reading and analysis.

In network maps, each term is represented by a circle whose size corresponds to its weight. The terms with highest occurrence form clusters, for better visualization of the existing research areas related to the topic. The first cluster (blue) represents the host plant (*Eucalyptus* spp.) and the insect (gall, wasp, blue chalcid). The second cluster (green) stands for management strategies (*L. invasa*, *Eucalyptus*, Hymenoptera, biological control). The third cluster (yellow) corresponds to the wasp physiology and origin of studies involving the topic (Figure 2).

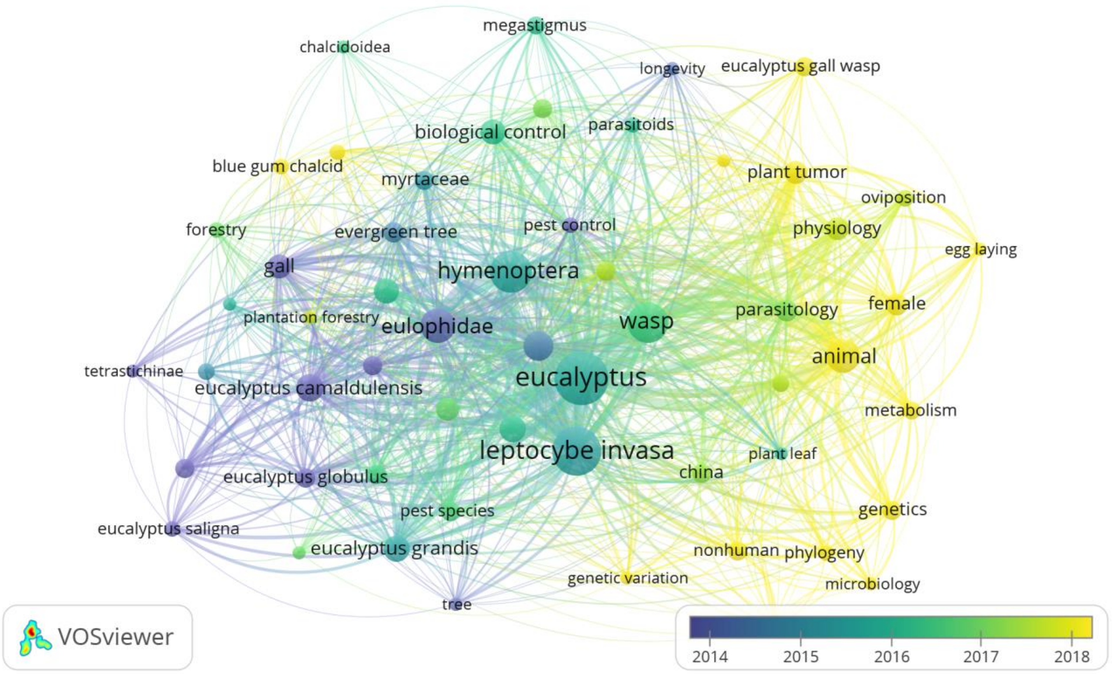


Figure 2 - Overlay visualization of keywords.
Source: VOSviewer 2023.

Based on the overlay map (Figure 2), the annual evolution of the research output can be shown by trend analysis. It was observed that the earliest studies focused on the identification and taxonomy of *L. invasa*, as well as reports of pest occurrence in *Eucalyptus* plantations (Mendel et al. 2004; Protasov et al. 2007; Kim et al. 2008; Nyeko et al. 2010; Zhu et al. 2012).

Different control methods were described in the studies, namely: biological control (Kim et al. 2008; Protasov et al. 2008; Sangtongpraow et al. 2011; Sangtongpraow and Charernsom 2013; Dittrich-Schroder et al. 2014; Ramanagouda and Vastrad 2015; Zheng et al. 2016; Hurley et al. 2017; Masson et al. 2017; Mendel et al. 2017; Barbosa et al. 2018; Ndlela et al. 2018; Gevers et al. 2021; Tavares et al. 2023), selection of resistant plant genotypes (Dittrich-Schröder et al. 2012), silvicultural management (Odhiambo et al. 2013; Roy and Mitra 2013; Zheng et al. 2016), integrated pest management (Ma et al. 2019), chemical insecticides (Javaregowda and Prabhu 2010; Vastrad et al. 2011; Roy and Mitra 2013; Roychoudhury 2018; Luna-Cruz et al. 2020), and tree breeding for resistance (Mphahlele et al. 2021).

Insecticide application for *L. invasa* control has received little attention in the studies, although some publications reported a somewhat successful impact of the method. So far, the active ingredients applied were Carbofuran, Imidacloprid, Spirotetramat, Thiamethoxam, Fipronil, Acetamiprid, Neonicotinoid, Dimethoate, Oxydemeton methyl, and Chlorpyrifos

(Luna-Cruz et al. 2020; Roy and Mitra 2013; Roychoudhury 2018; Vastrad et al. 2011). However, the use of these insecticides can make forest areas inappropriate for FSC certification The globally recognized standards for chemical pesticide hazard identification were extracted from a combination of sources, which include FSC Criteria. These criteria take multiple factors into account, e.g., the immediate harm caused by toxicity, enduring health risks (e.g., with regard to carcinogenicity, as warned by the Global Harmonized System of Classification and Labeling of Chemicals), impacts on reproduction, and mutagenic properties (FSC 2019a).

Carbofuran is an item in the FSC list of prohibited chemical pesticides. This catalogue comprises 48 pesticides, including heavy metals and dioxins. In addition, Imidacloprid, Chlorpyrifos, Fipronil, Acetamiprid, Dimethoate and Oxydemeton methyl are all included in the FSC list of restricted chemical pesticides (FSC 2019b).

Meanwhile, since 2014, long-term studies have been conducted to identify biological agents for *L. invasa* control. As a result, several parasitoids have been identified as promising candidates for an effective control of this pest (Table 1).

TABLE 1 - Parasitoid species used as biological control agents of *Leptocybe invasa*.

Family	Species	References
Eulophidae	*Aprostocetus causalis* La Salle & Wu, 2014	Zheng et al. 2016; Huang et al. 2018
	Aprostocetus gala (Walker, 1847)	Vastrad et al. 2009; Kulkarni et al. 2010
	Aprostocetus Westwood, 1833	Vastrad et al. 2009
	Quadrastichus mendeli Kim & La Salle	Kim et al. 2008; Protasov et al. 2008; Huang et al. 2012; Roy and Mitra and Mitra 2013; Klein et al. 2015; Nugnes et al. 2016; Zheng et al. 2016; Mendel et al. 2017; Bush et al. 2018; Kenis et al. 2019; Sangtongpraow and Charernsom 2019; Gevers et al. 2021; Puretz et al. 2022
	Selitrichodes kryceri Kim & La Salle, 1913	Kim et al. 2008; Protasov et al. 2008; Mendel et al. 2017; Kenis et al. 2019
	Selitrichodes neseri Kelly & La Salle, 1913	Kim et al. 2008; Kelly et al. 2012; Roy and Mitra et al. 2013; Dittrich-Schroder et al. 2014; Masson etal. 2017; Barbosa et al. 2018; Ndlela et al. 2018; Kenis et al. 2019; Gevers et al. 2021
Torymidae	*Megastigmus dharwadicus*	Ramanagouda and Vastrad 2015
	Megastigmus lawsoni Graham, 1969	Protasov et al. 2008; Mendel et al. 2017; Kenis et al. 2019
	Megastigmus manonae Bouček, 1988	Le et al. 2020
	Megastigmus pretorianensis	Le et al. 2020; Gevers et al. 2021

	Megastigmus sichuanensis Luo, 2001	Zheng et al. 2016; Huang et al. 2017; Huang et al. 2018
	Megastigmus thtipornae	Sangtongpraow and Charernsom 2013
	Megastigmus viggianii Narendran & Sureshan, 1988	Zheng et al. 2016
	Megastigmus zebrinus	Hernández et al. 2015; Klein et al. 2015; Gevers et al. 2021
	Megastigmus zvimendeli Van Noort & Pool, 2012	Protasov et al. 2008; Mendel et al. 2017; Kenis et al. 2019; Le et al. 2020
	Megastigmus Dalman, 1820	Vastrad et al. 2009; Tavares et al. 2023
Mymaridae	*Parallelaptera* Enock, 1909	Vastrad et al. 2009; Kulkarni et al. 2010
Platygasteridae	*Telenomus* Haliday, 1833	Kulkarni et al. 2010

These parasitoids of the order Insecta have been documented in various countries: *Aprostocetus gala*, *Quadrastichus mendeli*, *Selitrichodes kryceri* and *Selitrichodes neseri* in Australia; *Aprostocetus causalis* in Thailand; *Aprostocetus causalis* and *Megastigmus dharwadicus* in China; and *Aprostocetus gala* in India. In addition, *Quadrastichus mendeli* has been reported in Brazil, Italy and South Africa (Gbif 2023). The occurrence of these parasitoids in different countries indicates the possibility of widespread application in biological control strategies against *L. invasa*.

The parasitoids *A. causalis*, *Q. mendeli*, *S. kryceri* and *S. neseri* play a fundamental role in regulating *L. invasa* populations and mitigating their impact on *Eucalyptus* trees. The effectiveness of these species in controlling gall wasp has been demonstrated and they have been widely used in various biological control programs worldwide.

The results of the introduction and establishment of these parasitoids in affected areas have been promising in reducing pest infestation and minimizing economic losses in *Eucalyptus* plantations. However, it is worth mentioning the issues that arise with regard to breeding programs for these parasitoids for biological control. The sufficiency and timeliness of parasitoid availability and accessibility may be limiting factors for a successful implementation as biological control agents. Efforts are underway to address these challenges and develop strategies to overcome the limitations associated with the population size and distribution of these parasitoids for an effective management of *L. invasa*.

In addition, classical biological control has proven to be viable and sustainable option for managing the pest (Ndlela et al. 2018). Israel, located within the initial outbreak region, was the first country to launch a successful biological control program against *L. invasa,* based on Australian parasitoids such as *M. zvimendeli*, *M. lawsoni*, *S. kryceri* and *Q. mendeli* (Le et al. 2018). Current approaches, including chemical insecticides and biological agents, have had limited success in reducing infestation levels and mitigating the damage on *Eucalyptus* plantations.

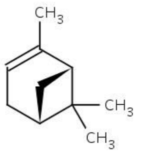
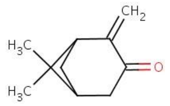
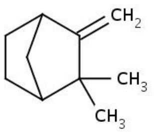
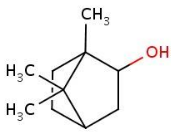
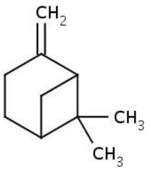
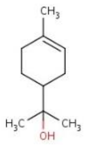
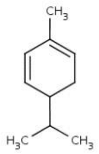
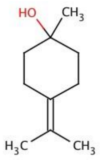
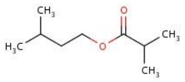
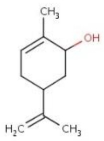
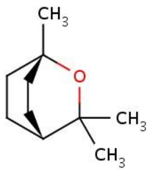
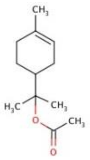
In 2016, the genes and metabolic compounds that may contribute to the varying tolerance levels of *Eucalyptus* clones to *L. invasa* were addressed in a number of studies (Mohamed 2016; Tong et al. 2016; Tobias et al. 2017; Li et al. 2017; Floris et al. 2018; Naidoo et al. 2018; Xiang et al. 2020; Mhoswa et al. 2020; Otieno et al. 2022; Pinsupa et al. 2023). These studies investigated the mechanisms underlying the differences observed in pest susceptibility, to elucidate possible genetic and metabolic factors that influence the interaction between *Eucalyptus* and *L. invasa*.

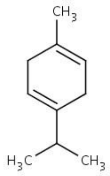
Genomic *loci* associated with resistance to gall-inducing insects in *Eucalyptus* species were identified for the first time in 2018. Favorable alleles are potential molecular markers in marker-assisted selection for gall wasp resistance (Zhang et al. 2018). Furthermore, these *loci* can be analyzed using bioinformatics tools to track genomic characteristics of interest, select markers for elite genotypes and investigate the events that induce resistance. The availability of genomic sequence data for *E. grandis* make them a promising tool for analyses of defense responses to current and emerging pathogens in various species of the Myrtaceae family (Christie et al. 2016).

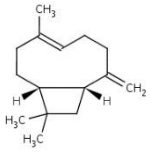
Based on transcriptome profiles of resistant and susceptible genotypes of an independent experiment, several putative candidate genes were identified in associated genomic *loci*, including Nucleotide-binding ARC-domain (NBARC) and tollinterleukin-1-receptor-Nucleotide binding signal Leucine-rich repeat (TIR-NBS-LRR) (Mhoswa et al. 2020).

Data pertaining to genes and secondary metabolites were extracted from articles documenting the presence of metabolites linked to gall-wasp tolerance. The primary compounds and their corresponding chemical structures were listed in Table 2.

TABLE 2 – Best-represented chemical structures of secondary compounds identified in *Eucalyptus* hybrids.

Compounds	Structure	Compounds	Structure
α-Pinene		Pinocarvone	
Camphene		Borneol	
β-Pinene		α-Terpineol	
α-Phellandrene		γ-Terpineol	
Isoamyl Isobutyrate		(Z)-Carveol	
1,8 Cineol		α-Terpinyl acetate	
Y-Terpinene		(E)-Caryophyllene	





Source: Dantas 2019; Mohamed 2016; Naidoo et al. 2018.

Previous studies have indicated that compounds such as α-terpineol, bicyclogermacrene and 1,8-cineole have insect-repellent properties (Oates et al. 2015). In addition, *L. invasa* infestation has been found to increase the number of certain chemical classes in gall-wasp-ridden plants, such as aldehydes, benzenoids, hydrocarbons, ketones and terpenoids (Huang et al. 2022).

Gall wasp attack on *Eucalyptus* trees leads to an increase in the leaf concentration of α-phellandrene. The biosynthesis of monoterpenes in *Eucalyptus* involves four metabolic pathways. Gall wasp infestation induces specific enzymes in the pinene and phellandrene pathways, as well as the terpinen-4-yl cation pathway, but inhibits enzymes in the α-terpineol pathway. As a result, there is a rise in phellandrene levels at the expense of oxygenated and aromatic monoterpenes (Mohamed 2016).

Significant differences were observed in the levels of amino acids and organic acids between resistant and susceptible *Eucalyptus* clones in response to oviposition. These findings suggest potential variations in metabolic profiles and defense mechanisms between the two groups (Oliveira et al. 2022).

A heat map was generated from percentage data to present the chemical components in *L. invasa*-resistant and susceptible *Eucalyptus* spp. genotypes. Parameters that were not originally expressed in percentage were converted by considering the highest values within the tolerance range and then represented as percentage (Figure 3).

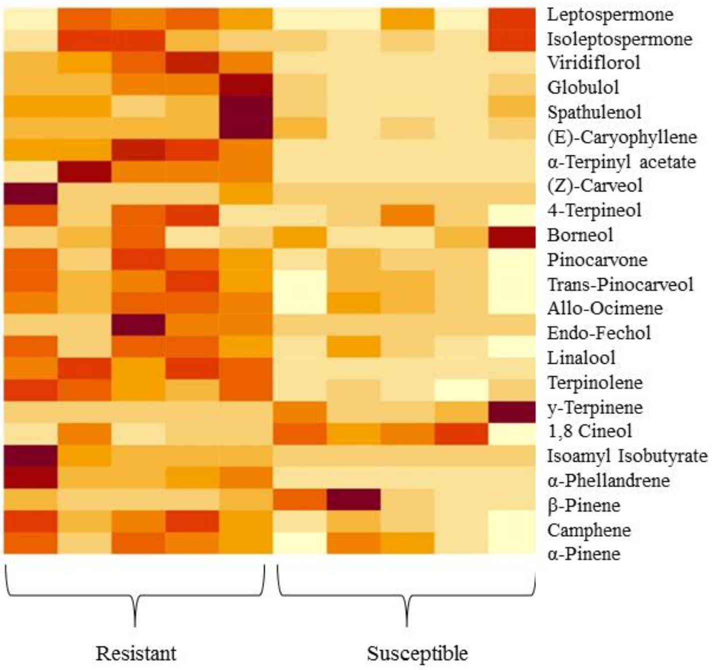


Figure 3 - Heat map of the chemical constituents in gall-wasp (*Leptocybe invasa* Hymenoptera: Eulophidae) -resistant and -susceptible genotypes of *Eucalyptus* spp.

The heat map clearly shows a relationship between compound concentration and tolerance. (E)-Caryophyllene, α-terpinyl acetate, (Z)-Carveol, Terpinolene, Isoamyl Isobutytrate and α-phellandrene were found to be present at higher concentrations in resistant genotypes (Oates 2015; Mohamen 2016; Huang 2022). Moreover, the studies claimed that α-phellandrene, linalool, α-terpinyl acetate and viridiflorol were produced by all tested *L. invasa*-resistant *Eucalyptus* genotypes.

Chemical constituents such as 1,8-cineole, α-pinene, α-terpineol, γ-terpinene, α-terpinyl acetate, trans-pinocarveol and borneol have been reported in *Eucalyptus* genotypes classified as resistant to *L. invasa* (Mohamed 2016). The variation in terpene traits, including 1,8-cineole, γ-terpinene and p-cymene, may be influenced by a few *loci* with minor and major effects on *L. invasa* resistance, indicating an oligogenic or possibly polygenic nature of these traits (Mhoswa et al. 2022). Variations in terpene profiles have also been observed between resistant and susceptible *Eucalyptus* genotypes in response to *L. invasa* oviposition (Oates et al. 2015; Naidoo et al. 2018).

Thus, this study used these findings to deepen the understanding of the genes involved in the response to *L. invasa* and the terpene pathway.

4.3.2 Data mining of genes and secondary compounds

Knowledge on the host range of natural enemies of *Leptocybe* spp. is important in evaluating a potential non-target impact on the local insect fauna and to understand interactions among gall associates of *Eucalyptus* (Le et al. 2018). Heavy infestation of the wasp results in vigor and growth loss and eventually in twig dieback. With the increasing pest threats to crops, the need for screening the existing germplasm and for new selections that are tolerant to *L. invasa* becomes urgent. Future breeding efforts should be focused on yield and tolerance against this severe pest. Another area where optimization is needed are enhanced management practices of insect pests (Kumar et al. 2015).

Certain genes have been identified as related to *L. invasa* resistance in *Eucalyptus* trees. Ten specific *Eucalyptus* genes were identified and analyzed in the Phytozome database to determine gene length, GO term and function (Table 3).

Table 3 - Gene name, length, GO term, definition and references of genes possibly associated with *Leptocybe invasa* tolerance.

Genes	Length	GO Term	Definition	References
Eucgr.B02310	2752	GO:0005524	Ribulose Bisphosphate Carboxylase/Oxygenase Activase, Chloroplastic	Pinsupa et al. 2023
Eucgr.F00808	2494	GO:0003743	Eukaryotic Translation Initiation Factor Sui1	
Eucgr.I00319	4795	GO:0005524	Abc Transporter F Family Member 2-Related	
Eucgr.C02944	2123	No Ontology data available	Plant Protein Of Unknown Function	Mhoswa et al. 2020
Eucgr.G00853	4748	GO:0008152	Riboflavin Kinase / Rfk	
Eucgr.G00856	2871	GO:0043531	Leucine-Rich Repeat-Containing Protein	
Eucgr.G00826	1371	No Ontology data available	Plant Protein Of Unknown Function	
Eucgr.G01032	472	No Ontology data available	Plant Protein Of Unknown Function	
Eucgr.H00321	1164	GO:0006032	Chitinase-Related	Tobias et al. 2017
Eucgr.C00395	2123	GO:0005975	Chitinase-Like Protein-Related	

Gene *Eucgr.H00321* is associated with the Chitin catabolic process, which involves the chemical reactions and pathways responsible for breaking down chitin, a linear polysaccharide composed of beta-(1->4)-linked N-acetyl-D-glucosamine residues. Genes associated with chitin decomposition can play a dual role in plant resistance to both insects and fungal pathogens (Osman et al. 2015). Gene *Eucgr.G00853* encompasses the broad range of chemical reactions and pathways that occur in living organisms. It includes anabolism and catabolism processes, which involve the transformation of chemical substances. These processes not only affect small molecules but also macromolecular processes such as DNA repair and replication, as well as protein synthesis and degradation.

Gene *Eucgr.I00319* and *Eucgr.G00856* represent the function of selectively and non-covalently interacting with ATP (adenosine 5'-triphosphate), which is a crucial coenzyme and enzyme regulator that plays a universal role in various cellular processes, an important molecule involved in cellular energy metabolism and signaling pathways. Lastly, gene *Eucgr.B02310* and *Eucgr.I00319* reiterate the function of selectively and non-covalently interacting with ATP (adenosine 5'-triphosphate), which served as a crucial coenzyme and enzyme regulator, playing a universal role in various cellular processes.

Eucgr.F00808 refers to the function of initiating ribosome-mediated translation of mRNA into a polypeptide. This process is fundamental in protein synthesis and involves ribosome translation of genetic information encoded in mRNA to synthesize a polypeptide chain. *Eucgr.C00395* encompasses the chemical reactions and pathways related to carbohydrates. Carbohydrates are organic compounds represented by the general formula $Cx(H_2O)y$. This term includes various processes involving carbohydrates, e.g., the formation of carbohydrate derivatives by the addition of a carbohydrate residue to another molecule.

In five cases (*Eucgr.C02944*, *Eucgr.G00853*, *Eucgr.00856*, *Eucgr.G00826* and *Eucgr.G01032*), gene expression was significantly different between resistant and susceptible samples. These genes encoded one protein of the TIR–NBS–LRR family, a riboflavin kinase/FMN hydrolase, an NB-ARC domain-containing disease resistance protein and two unknown plant proteins. The expression patterns of *Eucgr.G00826* and *Eucgr.G01032* were significantly different in response to oviposition between the resistant and susceptible samples, suggesting important roles in the outcome of the interaction (Mhoswa et al. 2020).

A study investigating the responses of *Eucalyptus* to gall wasp infestation revealed the up-regulation of genes *Eucgr.B02310, Eucgr.F00808* and *Eucgr.I00319* in all tested tolerant plants. This finding suggests the significant roles of these genes in the development of tolerance to gall wasp infestation (Pinsupa et al. 2023).

The terpene biosynthesis pathways involve both the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways. These pathways are responsible for generating a variety of terpenes in plants such as *Eucalyptus* trees. The specific processes and enzymes can vary depending on the terpene type and the organism (Gershenzon and Dudareva 2007).

Within the Plant Reactome Gramene Pathways, a pathway originating from the *Eucgr.B02310* gene has been elucidated. This gene is associated with terpene compounds. Functional analysis has revealed that *Eucgr.B02310* is part of a multifaceted pathway involving numerous enzymatic steps and regulatory components (Figure 4).

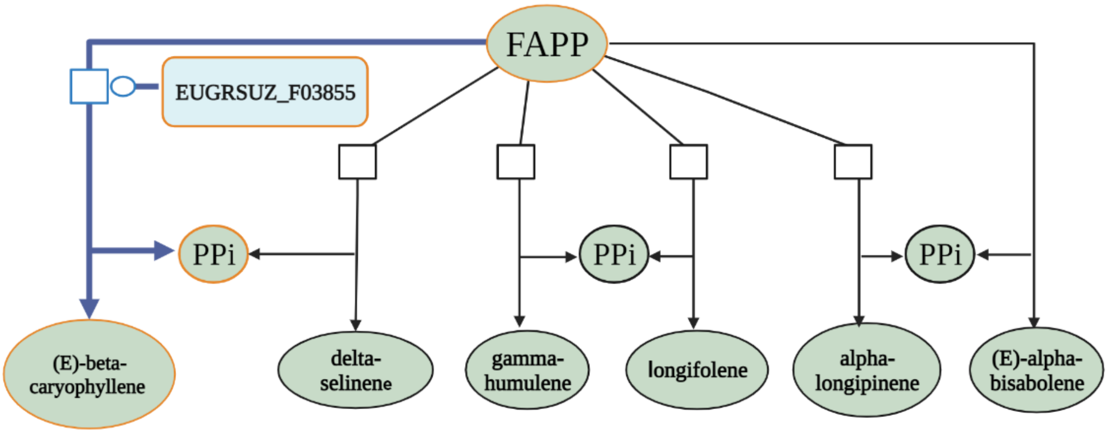


Figure 4 - Pathway of gene Eucgr.B02310 associated with the terpene compound. Source: Plant Reactome 2023.

The biosynthetic pathway of volatile sesquiterpenes, trans-(E)-beta-caryophyllene+diphosphate, involves the conversion of trans-farnesyl diphosphate (FPP) into (E)-beta-caryophyllene by the enzymatic activity of terpene synthase.

The mevalonate (MVA) pathway begins with the conversion of acetyl-CoA to mevalonate, which undergoes multiple enzymatic reactions that result in the production of pyrophosphate (PPi).

Pyrophosphate can then enter different branches of the terpene biosynthetic pathway, facilitating the synthesis of diverse terpene compounds, e.g., monoterpenes, sesquiterpenes and diterpenes.

Understanding the genetic factors underlying plant defense mechanisms is a promising approach to enhance *Eucalyptus* resistance against *L. invasa*. Molecular studies have identified potential genes and genetic pathways associated with resistance. These findings can be a guide for the development of new strategies of *L. invasa* control and contribute to sustainable forestry practices. A deeper understanding of the genetic basis of resistance can help to optimize the methods of combating this pest, to reduce reliance on harmful pesticides and preserve health and productivity of *Eucalyptus* forests.

4.3. Patent mining

A total of 40 patent documents were identified, of which only 20 have been published. Nine of them were filed by offices in China, six by the United States, four by the World Intellectual Property Organization and one by South Africa. These patents were filed in wide range of jurisdictions, demonstrating a global interest in the subject matter.

Technological innovations consist of the acquisition of new drugs to control pests such as *L. invasa*, as well as the development of engineered genes that could potentially be patented. An outstanding country in patent registrations is China, where intellectual innovations are captured (Rikap 2022). The main inventors are Avisar Dror, Shain Ziv, Stein Hanan and Siegel Hanan, with 14, 9, 9 and 6 patents, respectively.

The highest number of patents was filed in 2012 (10). However, only three patent documents were registered in that year. 2014 was the year with the highest number of published patent registrations (5), followed by 2012, 2015 and 2022, with the registration of three patents each (Figure 5). This interplay of patent publication and registration data is aligned with the occurrence records of the insect, suggesting a correlation between patent filings and pest pressure.

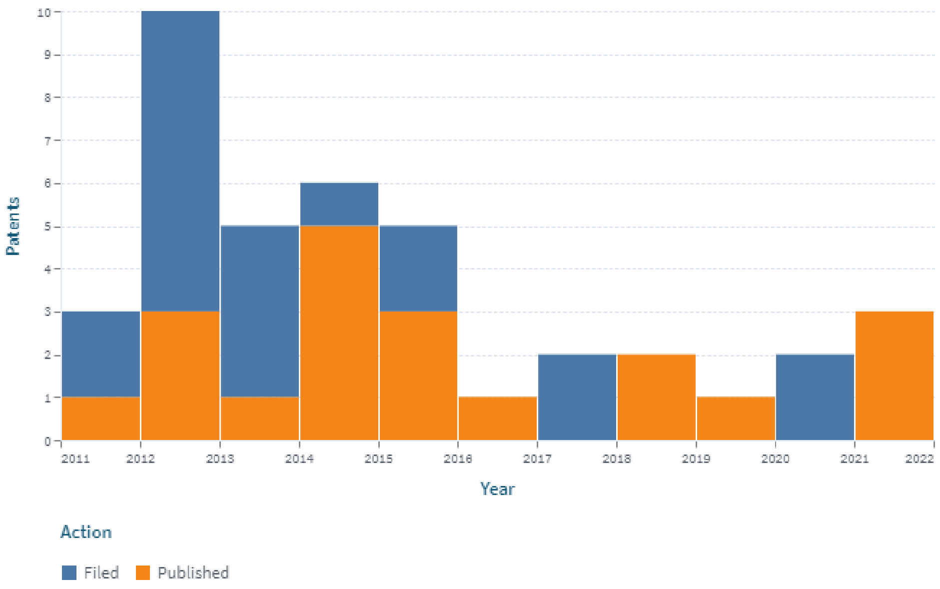


Figure 5 - Patent documents organized by publication and filing dates.

The landscape map for technology related to *L. invasa* control (Figure 6) showed the technological development for the control of the wasp across different areas such as agricultural pest control, phytopathogenic pests, agricultural chemical substances, olefin polymer technology and gene expression.

Clusters on the map have different colors, indicating various families (Figure 6). Each cluster represents an area with points of the same color, which correspond to a specific family. The relevant concepts are represented by the corresponding color. The distance between two points is proportional to their semantic concepts, resembling a topographic map.

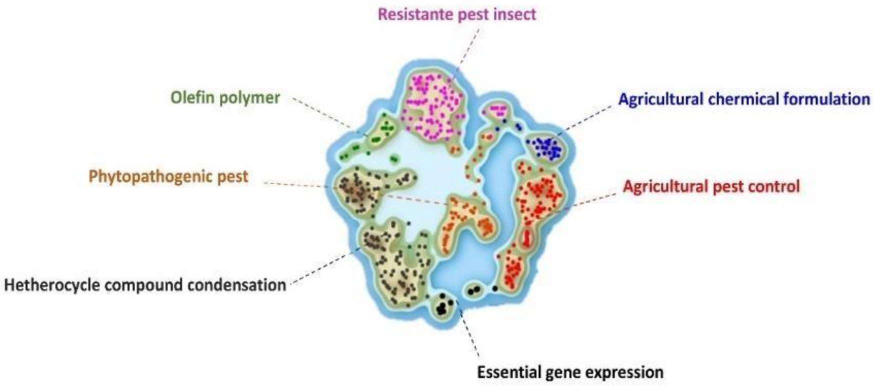


Figure 6 - Landscape map for technology related to *Leptocybe invasa* control.
Source: Questel Orbit® 2021.

Technological domains are defined based on patent classifications provided by the Cooperative Patent Classification (CPC). The main products focused on controlling the *Eucalyptus* gall wasp were categorized as follows:

A01N 43 - Biocides, pest repellants or attractants, or plant growth regulators containing substances (14 patents);

C12N 15 - Mutation or genetic engineering; DNA or RNA related to genetic engineering, vectors (e.g., plasmids), or their isolation, preparation, or purification; use of hosts for insect resistance (12 patents);

Y02A 90 - Technologies with an indirect contribution to adaptation to climate change, including monitoring or fighting invasive species (4 patents);

C12N 2310 - Structure or type of the nucleic acid (interfering nucleic acids) (4 patents);

C12N 9 - Enzymes; proenzymes; compositions thereof; Y02A 40 - Adaptation technologies in agriculture, forestry, livestock, or agro-alimentary production, including genetically modified (GMO) plants (e.g., transgenic plants) and C07K 14 - Peptides consisting of more than 20 amino acids, including gastrins, somatostatins, melanotropins and their derivatives (derived from wasps). These patent classifications provide an overview of the technological approaches and products developed for the control of the *Eucalyptus* gall wasp, with two patents in each classification (Figure 7).

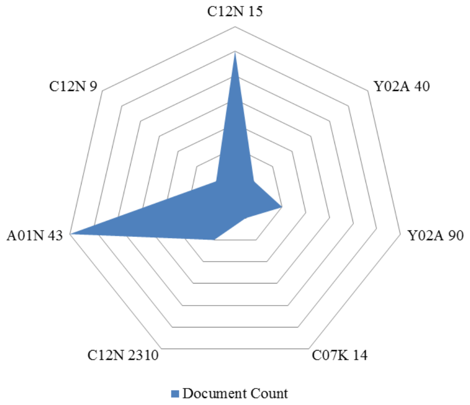


Figure 7 - Document count by Cooperative Patent Classification (CPC).

Insights into the biology and genetics of *L. invasa* may also contribute to the development of technological innovations, particularly in pesticides. However, these substances often have adverse environmental effects and sustainable technologies must be developed. Most patents related to *L. invasa* control involve the development of pesticides (Sikuljak and Gewehr 2014; James and Bristol 2016; Bristol 2018). One example is an insecticide composition containing spirotetramat and ivermectin (James and Bristol 2015).

One invention involves a volatile component that attracts *L. invasa* and traps it on a viscous plate. The *L. invasa* population can be monitored by the number of trapped insects. This solution is inexpensive, simple, practical and environmentally friendly (Yizhen et al. 2012).

Patents classified under "Mutation or genetic engineering; DNA or RNA concerning genetic engineering, vectors" have played a significant role in advances related to insect species. One notable development in this field is RNA-mediated gene silencing, which has been facilitated by gene sequencing from *Eucalyptus* sp., *L. invasa* (Li) and *Ophelimus maskelli* (Om) (Avisar et al. 2013). These patents have contributed to the understanding and utilization of genetic engineering techniques for insect control and management.

4.4. Conclusions

By scientific mapping, information about management strategies and the physiology and origin of *L. invasa* was found. Various control methods were discussed in detail, including biological control, selection of resistant planting stock, silvicultural management, integrated pest management and chemical insecticides. Promising parasitoids have been identified as potential biological control agents for *L. invasa* control.

Further research is needed to understand the genetic and molecular mechanisms underlying *Eucalyptus* resistance to the gall wasp. By gaining insights into these mechanisms and identifying the key genes, protein sequences and pathways, more targeted strategies can be developed to combat gall wasp infestations. This knowledge will have significant implications for sustainable *Eucalyptus* plantation management and the economic growth of the forestry sector.

Mining patent data has unearthed a wealth of innovative approaches to combat *L. invasa*. Scientific advancements have led to the identification of key genetic components and pathways associated with resistance to this pest.

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5. MANUSCRIPT 2

INSIGHTS INTO *Leptocybe invasa* RESISTANCE IN *Eucalyptus*: PHENOTYPING, GENOTYPING and *IN SILICO* APPROACHES

ABSTRACT

The gall wasp *Leptocybe invasa* poses a significant global threat to *Eucalyptus* cultivation, by causing substantial economic losses. The objective of this study was to differentiate between resistant and susceptible genotypes by morphological characteristics using image analysis based on the damage caused by the gall wasp. In addition, consensus sequences derived from transposable elements (TEs) and the genome of *Eucalyptus* spp. Were identified by *in silico* analysis. Furthermore, another objective was to discriminate *Eucalyptus* genotypes in response to *Leptocybe invasa* by conducting molecular analyses involving transposable elements and inter simple sequence markers. For image analysis, the GroundEye ® system was used to collect images of 60 leaves from six genotypes, three of which were resistant and three susceptible. *Eucalyptus* spp. sequences were obtained from the GenBank database by *in silico* analysis and pairwise alignments with TE sequences were conducted using BLASTN. Multiple sequence alignment was performed with Clustal Omega, followed by the identification of conserved regions in Jalview. A motif signature was generated using Weblogo. For molecular characterization using ISSR markers and TEs, samples of young leaves were obtained from a total of 80 *Eucalyptus* seedlings, of which 50 were classified as resistant and 30 as susceptible to *L. invasa*. It was possible to distinguish gall wasp susceptible and resistant genotypes by image analysis. *In silico* analysis enabled the identification of conserved regions in the *Eucalyptus* spp. genome, which were associated with proteins involved in secondary metabolite production, e.g., terpenes, which play a role in the response to *Leptocybe invasa*. The discrimination capacity of TEs and ISSR primers was demonstrated and bands were generated that could be used to identify resistant genotypes. However, increasing the number of markers required to discriminate genotypes in both cases is suggested.

Keywords: BLAST; terpene; economical damage; genetic diversity; Groundeye®; pest management.

5.1. Introduction

The gall wasp *Leptocybe invasa* represents a significant global threat to *Eucalyptus* trees, leading to substantial economic losses (Csóka et al., 2017; Le et al., 2018; Mendel et al., 2004). This pest primarily affects young plantations and causes devastating damage to seedlings in forest nurseries (Naidoo et al., 2018). The attack of *L. invasa* results in severe structural damage to *Eucalyptus* leaves, petioles and branches, affecting photosynthesis and forest yield (Carvalho et al., 2022; Rocha et al., 2023). Therefore, understanding this pest and establishing effective control strategies will be decisive to minimize productivity losses.

Studies that evaluated the response of *Eucalyptus* clones to *L. invasa* indicate that clones of certain pure species, including *Eucalyptus moluccana*, *Eucalyptus urophylla*, E. *camaldulensis*, *Eucalyptus dunnii*, *Eucalyptus nitens*, *Eucalyptus grandis*, as well as the hybrids *E. urophylla* × *E. grandis*, *E. urophylla* x *Eucalyptus* spp., *Eucalyptus saligna* × *E. urophylla*, can serve as sources of resistance to this pest (Dantas et al., 2021; Dittrich-Schröder et al., 2012; Eskiviski et al., 2018; Otieno et al., 2022). Dantas (2019) identified variations in the composition of secondary compounds, with a higher concentration of terpenes, among genotypes resistant and susceptible to the pest.

In integrated pest management, various measures can be adopted to deal with insects considered pests. This includes identifying the economic damage threshold, implementing cultural control measures, using biological and chemical control methods and utilizing resistant genotypes. In this regard, early evaluation of damage to vegetative structures can help identify the optimal timing for control by the use of chemical products. Tools that facilitate damage observation and monitoring, e.g., image analysis, are relevant to accelerate decision-making.

Genetic diversity plays a vital role in the adaptation and resilience of plant species to various stresses (Salgotra and Chauhan, 2023). The genetic composition of *Eucalyptus* genotypes can influence their ability to tolerate or resist *L. invasa* infestation (Mhoswa et al., 2022). Therefore, it is essential to evaluate the genetic distinction among *Eucalyptus* genotypes to identify resistant individuals that can contribute to breeding programs and the development of resistant clones.

To improve the understanding of genetic mechanisms in plants, a comprehensive analysis of genetic variants is necessary. The inbreeding level in the genus *Eucalyptus* is high. Multi-allelic markers, such as those provided by Transposable Elements (TEs), can be used as genetic markers to differentiate genotypes (Candotti et al., 2023; Wang et al., 2023). Transposable Elements are widely distributed in the *Eucalyptus* genome and influence various aspects of plant development and stress response. However, their specific involvement in the response to *L. invasa* in *Eucalyptus* remains unknown (Ferguson et al., 2023). Understanding the distinction of TEs between resistant and susceptible *Eucalyptus* plants can contribute to the selection of genotypes with desirable traits. Transposable Elements can also serve as potential molecular markers to measure genetic variability among different genotypes.

Inter-Simple Sequence Repeat (ISSR) markers have emerged as powerful tools for genetic diversity analysis (Sadeghpoor et al., 2023). They provide valuable information about the genetic structure and diversity within plant groups and are widely used in genetic studies due to their high variability and ability to detect polymorphisms (Shahabzadeh et al., 2020). ISSR markers are commonly used to discriminate genotypes (Ashraf et al., 2016; Basha and Sujatha, 2007; Biswas et al., 2010; Costa et al., 2016; Kanbar, 2011; Sharma et al., 2009), facilitating the identification of genotypes of interest.

The objective of this study was to differentiate between resistant and susceptible genotypes based on image analysis of damage caused by the gall wasp. In addition, consensus sequences derived from transposable elements and the *Eucalyptus* spp. genome were identified by *in silico* analysis. Moreover, the *Eucalyptus* genotypes in response to *Leptocybe invasa* was discriminated by molecular analyses that involve TEs and ISSR markers.

5.2. Material and Methods

5.2.1. Plant material

A total of 150 *Eucalyptus* seedlings were provided by Bracell Ltda for the experiment. After approximately 12 months, 80 seedlings were transplanted into 20 L containers filled with a substrate mixture of sand, coconut powder and goat manure (3:1:1). From these plantlets, samples of young leaves were collected for DNA extraction. Eighty seedlings were classified for resistance to *Leptocybe invasa,* according to Classification Table 1, proposed in earlier studies, wherein the Fournier index of 1975 was used (Dantas, 2019; Dantas et al., 2021).

Table 1 - Classification of *Eucalyptus* spp. genotypes based on their response to *Leptocybe invasa* infestation, with: resistance; low susceptibility; moderate susceptibility; and very high susceptibility.

Genotype	Phenotype	Female parent	Male parent	Origin
1404*	Resistance	*E.urophylla*	*Eucalyptus* spp.	11°35'56"S 38°36'39"W
1249*	Resistance	*E. grandis*	*E.urophylla*	12°45'47"S 38°20'34"W
1250*	Resistance	*E. grandis*	*E. urophylla*	12°45'47"S 38°20'34"W
0321*	Resistance	*E. grandis*	*E. urophylla*	11°56'45"S 38°04'52"W
5341*	Resistance	*Eucalyptus* spp.	*Eucalyptus* spp.	19°49'16"S 40°16'34"W
1277*	low susceptibility	*E. grandis*	*E. camaldulensis*	11°35'56"S 38°36'39"W
1262*	moderate susceptibility	*E. grandis*	*E. urophylla*	12°45'47"S 38°20'34"W
1275*	low susceptibility	*E. camaldulensis*	*Eucalyptus* spp.	11°35'56"S 38°36'39"W
1724	moderate susceptibility	*E. urophylla*	*Eucalyptus* spp.	-
0292	very high susceptibility	*E. grandis*	*E. urophylla*	11°56'45"S 38°04'52"W

*Genotypes used in the experiment.

5.2.2. Phenotyping

One-year-old *Eucalyptus* genotype seedlings under *L. invasa* attack were placed in paper bags and pressed for 48 hours to obtain flat surfaces to take two-dimensional images. For the analysis, a total of 60 leaves of six genotypes were evaluated.

The leaves were arranged in an acrylic tray in groups of 10 and images were captured from both the abaxial and adaxial leaf surfaces. The software captured a total of 453 geometric and morphological characteristics, including leaf area, diameter, elongation, perimeter, circularity, color and texture.

To calibrate the GroundEye® system, the YCbCr color model was employed, with luminance 0 - 1.0, blue values ranging from 0.07 to 0.5 and red values from -0.5 to 0.5. After background color calibration, the images were further analyzed.

Genetic diversity was measured based on Mahalanobis' Euclidean distance for the variables leaf area, contour deformation, maximum diameter, minimum diameter, irregularity, perimeter, contrast, dissimilarity and homogeneity using R software (R core Team, 2020).

The formula for the Mahalanobis distance is as follows:

$$D^\wedge 2 = (x - \mu)^\wedge T * C^\wedge(-1) * (x - \mu)$$

Where:

D is the squared Mahalanobis distance;

x the data vector of a specific point;

μ the mean vector of multivariate distribution; and

C^(-1) the inverse of the covariance matrix of the multivariate distribution.

The contribution of the variables to genotype distinction was assessed by principal component analysis (PCA), using R software (R core Team, 2020). Mean values were used to construct a correlation matrix, from which standardized principal component (PC) scores were derived. The relationships between the variables were examined by correlation analyses.

5.2.3 *In silico* study

Data retrieval

Genome sequences from seven eukaryotic organisms were used: *E. camaldulensis* Dehnh., *E. urophylla* S.T.Blake, *E. tereticornis* Sm., *E. globulus* Labill., *E. marginata* Sm., *E. melliodora* A. Cunn. ex-Schauer and *E. grandis* W. Hill ex Maiden. These sequences, either partially or fully sequenced, were obtained from the GenBank database.

Transposable element sequence and in silico analysis

Transposable elements (TEs) were analyzed in the genomes of *Eucalyptus* spp. using known TE sequences from genes (*CLIRAP1*, *CLIRAP4*, *SSR*, *URP13R*, *URP17R*, *URP2R*, *URP6R)*. These reference sequences were pairwise aligned and analyzed by BLASTn comparison.

Sequences producing significant alignments

The parameters set for this analysis were as follows:

1. TE copies must not exhibit long segmental changes with a continuous mismatch count of > 10 and,

2. TE copies must have an E-value of < 1E-10.

By applying these parameters, a list of TE sequences was obtained comprising individual TE copies from each genome.

Multiple sequence alignment

The TE sequences identified were aligned using Clustal Omega, which can be accessed at https://www.ebi.ac.uk/Tools/msa/clustalo/ (Sievers and Higgins, 2014). The alignment was visualized and conserved regions were identified with Jalview, a software tool (Waterhouse et al., 2009). The FASTA alignment of the protein sequence was done with MEGA11 software (Tamura et al., 2021). A motif signature was generated using Weblogo (Crooks et al., 2004). The computational structure to analyze the TE sequences is illustrated in Figure 1.

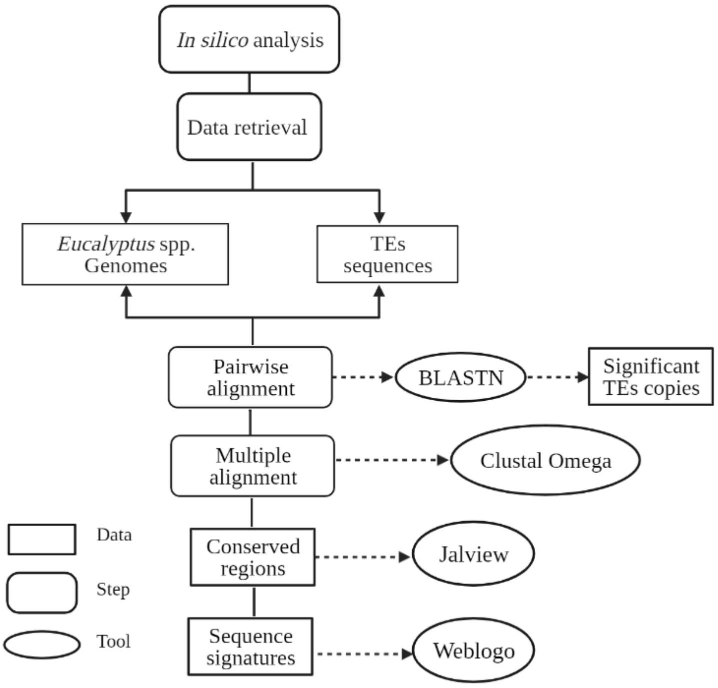


Figure 1 - A simplified computational framework based on bioinformatics was employed for the analysis of transposable element (TE) sequences in *Eucalyptus*.

5.2.4 Genotyping

Collection of genetic material

Young leaf samples were taken from a total of 80 *Eucalyptus* seedlings (Figure 2), of which 50 plants were classified as resistant and 30 plants as susceptible to *L. invasa*. These samples were stored at -20°C for TE and ISSR marker analysis.

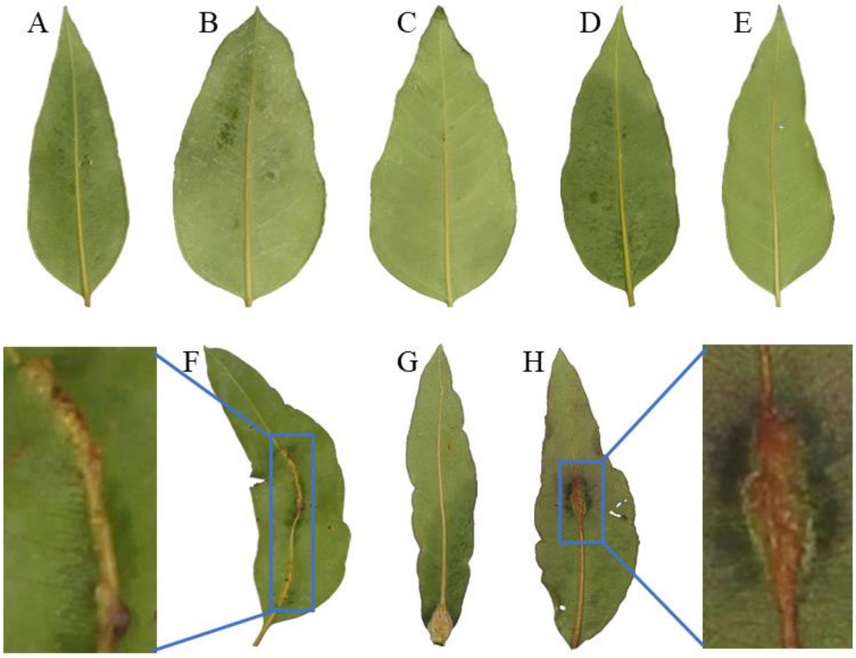


Figure 2 - A-H: *Eucalyptus* spp. leaf (abaxial view). Genotypes A (1404), B (1249), C (1250), D (0321) and E (5341) are resistant to *Leptocybe invasa* (Fig. A-E). Genotypes F (1277), G (1262) and H (1275) are susceptible to *L. invasa* (Fig. F-H).

DNA isolation

The DNA from young leaves of each genotype was extracted by a modified Cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987). Approximately 200 mg of leaf tissue was ground with 900 µL of 2% CTAB buffer and 50 µL of 0.2% β-mercaptoethanol. The samples were heat-treated at 65°C for 30 min and then purified by adding chloroform:isoamyl alcohol. After centrifugation, DNA was transferred to a new tube and precipitated using ammonium acetate : alcohol. The DNA pellet was washed, air-dried and resuspended in 70% ethanol. Additional washing with ethanol was performed and the DNA was finally solubilized in a 2-amino-2-hydroxymethyl-1.3-propanediol (Tris) - ethylene diamine tetraacetic acid (EDTA) buffer. The DNA quality and concentration were assessed with a spectrophotometer. The DNA was resuspended at 80 ng/µL and stored at -20°C.

Genomic DNA quantification

The quality and concentration of DNA samples were assessed using an Epoch® spectrophotometer. Absorbance readings at 260 nm and 280 nm were used for this analysis. To determine the concentration, 1.6 µL of TE buffer was aliquoted into the first well as a blank, while the remaining wells received 1.6 µL of DNA sample from the eight genotypes. The DNA samples were then diluted to a working concentration of 80 ng/µL.

PCR reaction

The target region of the DNA template was amplified using Platus Taq DNA Polymerase. The PCR reactions included 0.3 µL of *Taq* polymerase, 5 µL of 10X buffer with KCl, 2.5 µL of $MgCl_2$, 0.5 µL of dNTPs, 2 µL of primers, 4 µL of DNA and 5.7 µL H_2O.

The thermal cycling protocol consisted of initial denaturation at 95°C for 3 min, followed by 35 denaturation cycles at 95°C for 30 sec, annealing at 43-64°C for ISSR markers and 48-67°C for TEs (Table 2) and an extension step at 72°C for 1 min. A final extension was performed at 72°C for 10 min.

Table 2 - Transposable elements (TEs), ISSR primer sequences and annealing temperature (Ta).

Transposable elements (TEs)		
Primer	Sequence 5' - 3'	Ta °C
CLIRAP1	CGTACGGAACACGCTACAGA	67
CLIRAP4	CTTTTGACGAGGCCATGC	50
SSR	GAGAGAGAGAGAGAGAG AC	60
URP13R	TACATCGCAAGTGACACACC	48
URP17R	AATGTGGGCAAGCTGGTGGT	74
URP2R	CCCAGCAACTGATCGCACAC	65
URP6R	GCAAGCTGGTGGGAGGTAC	65
ISSR marker		
Primer	Sequence 5' - 3'	Ta°C
DAT	GAGAGAGAGAGAGARG	43ºC
UBC 834	AGAGAGAGAGAGAGAGYT	47ºC
Goofy	GTGTGTGTGTGTGTYG	48ºC
UBC809	GTGTGTGTGTGTGTYG	48ºC
Pat 1 - QF	GGTTCCATTGCTTACCGATT	60ºC
Pat 1 - QR	CAAGTTTCGAGGAACAGC	60ºC
Lxx22R	CAAGCAGGGGTACTAACTTCGA	64ºC
Cxx2	ACCCTGTGTTGTTTTCAACG	57°C
M2	GGGCAGAGAGAGAGAGAGAG	64ºC
UBC810	GAGAGAGAGAGAGAGAT	43ºC
UBC808	AGAGAGAGAGAGAGAGC	47ºC

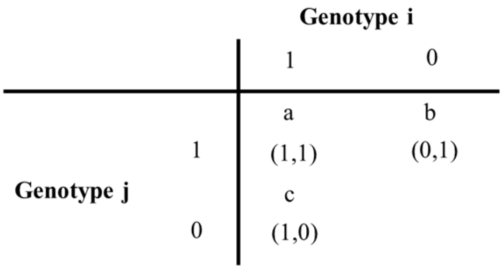
*Long Terminal Repeat (LTR) - *CLIRAP1, CLIRAP4* and *SSR*; Universal Rice Primers (URP).

The presence of amplified fragments was confirmed by agarose gel electrophoresis on 1.5% gel. Electrophoresis was carried out in 1% TBE buffer for 50 min at 100 V, 100 mA and 100 W. Safer dye® was used to stain the fragments, which were visualized under ultraviolet light in a transilluminator (LPIX, Loccus biotechnology).

Data analysis

Private fragments (bands) were quantified and genetic distances were estimated. Weakly stained and poorly defined fragments were excluded. The polymorphism percentage was determined and a binary matrix of presence (1) and absence (0) was constructed. The statistical software GenAlEx (Smouse et al., 2012) was used.

The genetic dissimilarities (dij) between each pair of genotypes were estimated using the Jaccard coefficient, expressed as $dij = b + c / a + (b + c)$. The variables in the expression were calculated as shown in the following diagram:



		Genotype i	
		1	0
Genotype j	1	a (1,1)	b (0,1)
	0	c (1,0)	

A simplified representation of genetic distances was achieved using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method in the DARwin software version 6.0.021 (Perrier and Jacquemoud-Collet, 2019).

5.3. Results and Discussion

5.3.1. Phenotyping

The results of the damage analysis revealed a more uniform texture of the resistant genotypes, indicating a smoother and more regular surface, compared to susceptible genotypes. In addition, the gap area of the resistant genotypes was smaller, suggesting a more compact leaf structure. On the other hand, susceptible genotypes had a larger gap area and deformed leaves, indicating a less uniform and more irregular surface (Figure 3).

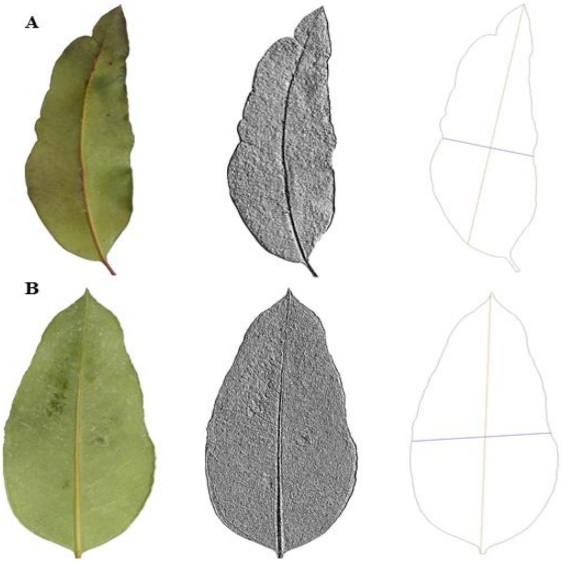


Figure 3 - GroundEye® images of a susceptible genotype to *L. invasa* (Genotype A) and a resistant genotype (Genotype B): Leaf image (back), Texture representation and geometry representation images.

Leptocybe invasa induces hyperplasia of leaf midribs, petioles and twigs of up to 2-year-old *Eucalyptus* seedlings and trees, and this deformation results in bump-shaped galls. Gall formation by *L. invasa* on growing shoot tips and leaves of *Eucalyptus* accelerates leaf abscission and causes shoot drying. A heavy infestation of the wasp results in loss of vigor and growth, which subsequently results in twig dieback (Kumar; Sangha; Dhillon, 2015).

Gall formation is the main symptom of the insect attack, and can be helpful in identifying the occurrence of the insect on trees, to define effective strategies to reduce the insect population in the plantation (Ortiz et al., 2017).

Figure 4 shows varying degrees of deformation on leaves of plants affected by gall wasps, indicating the presence of galls in different areas of the leaf. This observation confirms similar findings in previous studies. Compared to resistant genotypes, the levels of leaf deformation on susceptible genotypes infested with gall wasps were higher. These results further emphasize the susceptibility of certain genotypes to gall wasp damage and underscore the importance of identifying and exploiting resistant genotypes in breeding programs and pest management strategies.

Branch deformation was observed, in agreement with Dantas et al. (2021), indicating high *L. invasa* infestation in the petiole-midrib axis of leaves, petioles and young branches. This infestation can disrupt apical dominance and lead to branching or increased sprouting.

In the context of susceptibility to gall-inducing insects in planted forests, knowledge about morphological responses of hosts is still limited (Sarmento et al., 2021). The presence of *L. invasa* eggs on plant tissues leads to gall formation, leaf curling and premature leaf aging (Figure 4). Severe infestation can result in shoot death, leaf drop, stunted growth and weaken the tree, causing significant losses in *Eucalyptus* plantations.

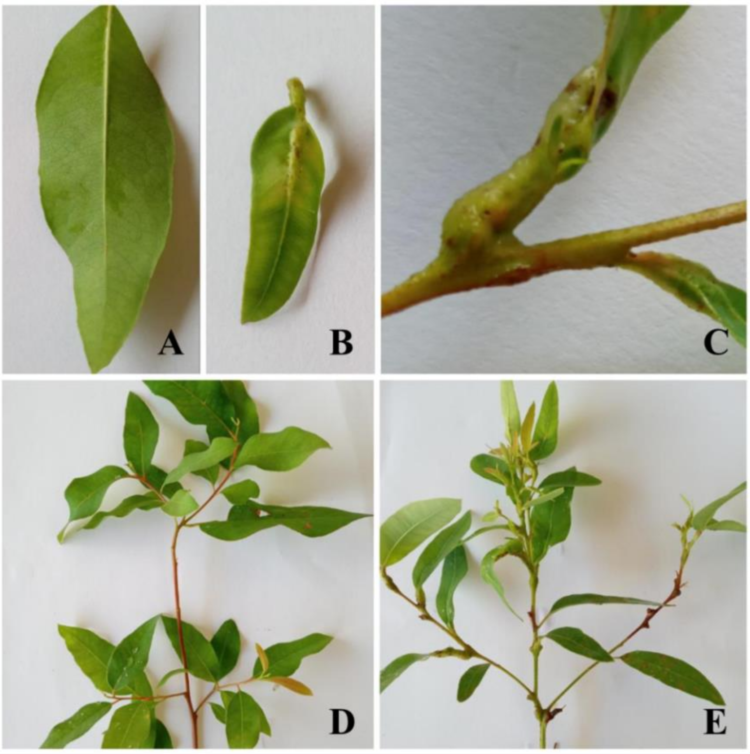


Figure 4 - Genotypes of *Eucalyptus* spp. with different responses to *Leptocybe invasa* attack. A: Gall-free new leaf of resistant genotype. B: Galls on new leaf of susceptible genotype. C: Galls on branches of susceptible genotype. D: Branch of resistant genotype. E: Branch of susceptible genotype.

The geometric variables, particularly leaf area and gap area, were found to be the most relevant in explaining the statistical variation. This supports previous studies (Mendel et al., 2004; Tong et al., 2016) that demonstrated the correlation between leaf size and surface damage changes caused by oviposition. These parameters indicate the level of pest activity and plant tissue degradation.

Based on quantitative analyses of damage data, a dendrogram was constructed (Figure 5), which facilitated the identification of two distinct groups: Group I, comprising three resistant genotypes (1404, 1249 and 1250) and Group II, consisting of three susceptible genotypes (1262, 1275 and 1277). Within Group I, genotypes 1404 and 1249 were most similar to each other, while differing from genotype 1250. In contrast, within Group II, genotypes 1277 and 1262 were most similar to each other, but differed from genotype 1275.

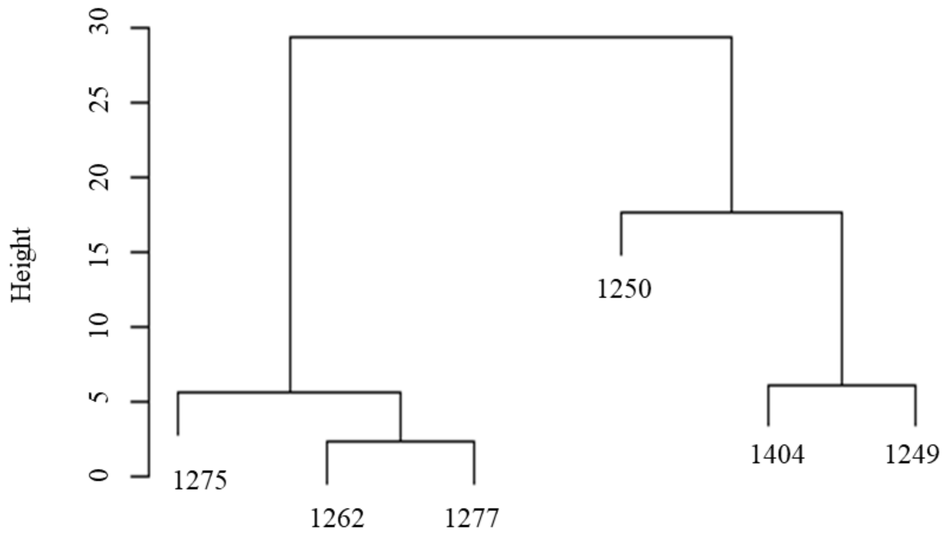


Figure 5 - Dendrogram of genetic relationships among *Eucalyptus* genotypes using Mahalanobis distances derived from phenotypic data.

The dendrogram provides insights into the genetic relationships and clustering patterns among the genotypes. Mahalanobis distance analysis demonstrates how damage traits can be used as informative markers for genotype selection. The dendrogram highlights the genetic dissimilarity between the susceptible and resistant genotypes, indicating distinct damage characteristics associated with their response to the pest. The clustering pattern suggested that these genotypes share similar genetic traits related to their susceptibility or resistance.

Based on phenological analysis data obtained from GroundEye, genotypes 1262, 1275 and 1277 were susceptible and genotypes 1249, 1250 and 1404 resistant. Phenological analysis not only corroborated the data acquired by Dantas (2019), but also enabled a precise differentiation of multiple phenotypes between resistant and susceptible genotypes. This innovation facilitates the identification of genotypes by more objective methods.

Principal component analysis – PCA was employed to identify the most important variables of the data set (Figure 6).

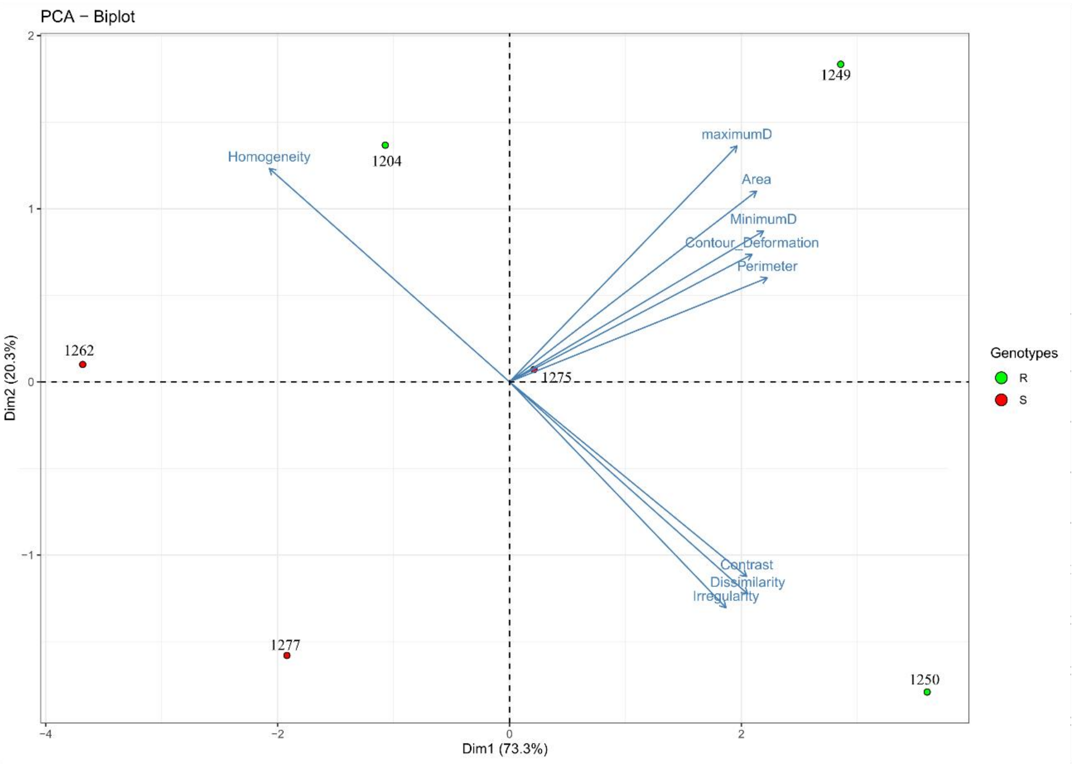


Figure 6 - Biplot obtained by the linear combination of variables related to the phenotypic variables of susceptible (S) and resistant (R) *Eucalyptus* genotypes.

The PCA analysis reduces the number of effective parameters to differentiate genotypes. In addition,leading traits that account for higher fractions of overall variability are summarized and identified and groupings that could not possibly emerge from the raw data are visualized. In this study, PCA, based on a correlation matrix, was conducted to determine the distinction among genotypes. Two principal components, namely "leaf area" and "contour deformation," were identified, together accounting for 93.6% of the overall variability.

The initial component, denoted as "leaf area," accounts for 73.3% of the variance, which means that these characteristics have the most significant variability across the genotypes and the greatest influence on their differentiation. The principal loadings on the PC1 axis were associated with irregularity, contrast and dissimilarity, as these factors had the greatest values and were key for genotype distinction. On the other hand, the secondary component "contour deformation" explained 20.3% of the variance. For the second principal component (PC2), the variable with the highest loading was homogeneity.

Leaf area, contour deformation, maximum diameter, minimum diameter and perimeter represented the greatest value on the PC1 and PC2 axis in two components. The remaining components explained less variability. These eigenvalues and their percentages provide insights into the contribution of each axis in explaining the dissimilarity among the factors under study. Higher eigenvalues indicate axes that capture more variation (Nguyen and Holmes, 2019; Stewart et al., 2014).

Figure 7 shows the correlation of variables. Leaf area was positively correlated with contour deformation (r = 0.89), maximum diameter (r = 0.94), minimum diameter (r = 0.94) and perimeter (r = 0.94). Also, leaf area was significantly positively correlated with irregularity (r = 0.49), contrast (r = 0.6) and dissimilarity (r = 0.7). These parameters can therefore be used to predict each other. Meanwhile, a negative correlation was found between homogeneity and all other variables.

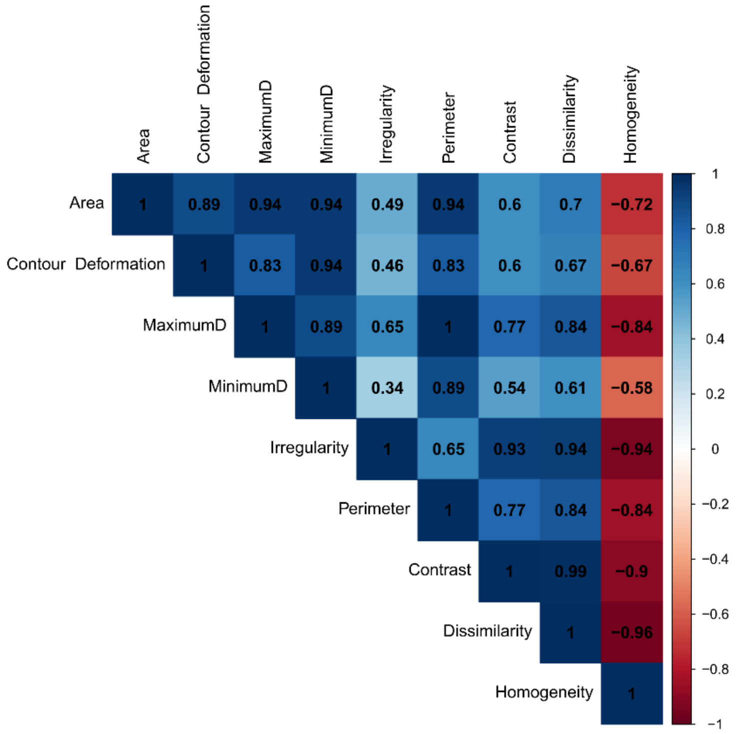


Figure 7 - Correlations between phenotypic variables of susceptible and resistant *Eucalyptus* genotypes.

The correlation coefficient can provide information on the most relevant traits to assess genotypes. These parameters can be used to predict others and could be taken into consideration for the characterization of genotypes. It is worth emphasizing that phenotypic analysis is considered a first step in the assessment of genetic diversity of a plant species (Hashemi and Khadivi, 2020).

5.3.2 *In silico* study

To analyze sequence conservation, multiple sequence alignment (MSA) was performed by the EMBL-EBI Clustal Omega server. The MSA included seven sequences, with lengths from 75 to 224 nucleotides, and a mean length of approximately 144 nucleotides. By employing appropriate alignment parameters and a substitution matrix, conserved regions, structural characteristics and sequence similarities among the TE copies were identified. The MSA analysis also facilitated the construction of a dendrogram, which represents the phylogenetic relationships among the sequences, providing insights into evolutionary relationships and divergence. The cladogram showed branching patterns, without including branch length information (Figure 8).

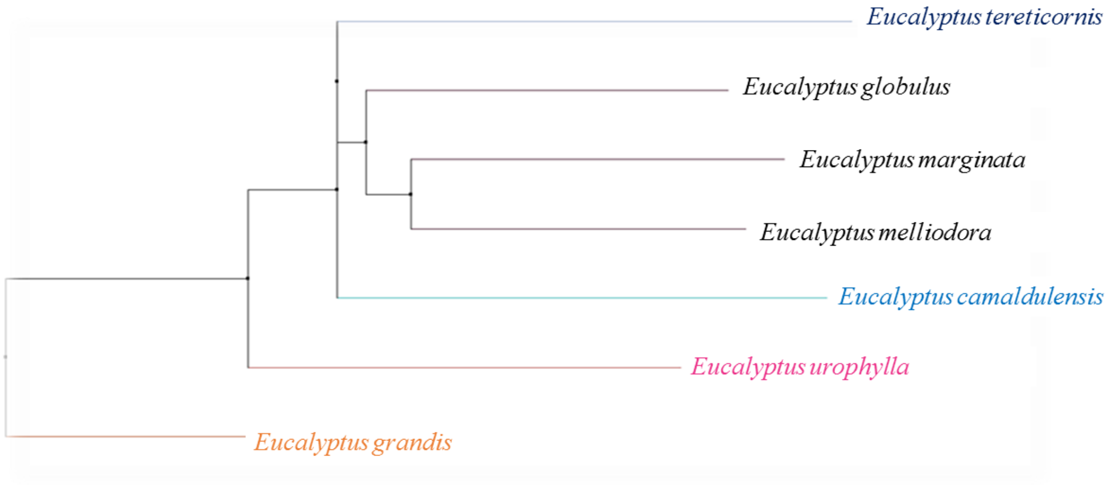


Figure 8 - Clustal Omega analysis with transposable element and genome sequences of *Eucalyptus* spp.

An analysis of the phylogenetic tree revealed the following relationships among the *Eucalyptus* sequences: a close relationship between *E. melliodora* and *E. marginata*, with a branch length of 0.34. These two sequences clustered together with *E. globulus*, which had a slightly shorter branch length (0.31). Within this cluster, the sequence of *E. tereticornis* diverged earlier, with a branch length of 0.29. On a separate branch, *E. grandis* branched off from the main cluster, with a slightly shorter branch length (0.24).

For *E. urophylla*, the sequences shared a branch length of 0.16. The phylogenetic tree structure indicated a close relationship between *E. melliodora*, *E. marginata* and *E. globulus*, while *E. grandis* branched off earlier. *E. urophylla*, *E. marginata* and *E. grandis* formed a distinct group (Figure 9).

Figure 9 - Neighbor-joining phylogram without distance corrections. Clustal Omega analysis with transposable element and genome sequences of *Eucalyptus* spp.

Eucalyptus melliodora and *E. marginata* were closely related (0.23 and 0.24, respectively). These two sequences clustered together with *E. globulus*, with a branch length of 0.13. Another cluster was formed by these three sequences, with a genetic distance of 0.065. *Eucalyptus urophylla* diverged from the main cluster (0.17). The Percent Identity Matrix analysis revealed varying degrees of similarity among the sequences. Highest similarity was observed between *E. camaldulensis* and *E. globulus* (70.33%) and the lowest between *E. grandis* and *E. camaldulensis* (39.66%).

The *L. invasa*-resistant genotypes used in this study for analysis with TE and ISSR markers were hybrids between *E. urophyla* and *E. grandis.* Retrotransposons accounted for the major portion of the *E. grandis* genome (44.5%), while long terminal repeat retrotransposons were the best represented class (21.9%). The DNA transposons encompassed only 5.6% of the genome. For this class, Helitron elements were the most abundant, with an estimated 15,000 copies or 3.8% of the genome (Myburg et al., 2014).

Multiple sequence alignment generated by Clustal Omega revealed conserved patterns and variations within the TE sequences, indicating the significance of these regions in *Eucalyptus* genomes. Conservation was high in certain regions, particularly among *E. marginata*, *E. melliodora* and *E. globulus*, suggesting a close phylogenetic relationship. On the other hand, significant variations were observed among *E. grandis*, *E. tereticornis*, *E. urophylla* and *E. camaldulensis*, indicating higher diversity at these *loci* (Figure 10).

TGTTCCGTACCGCTACAGATACGGAACATGGC
CTTGACGACGACATGGTAGGCATCGTCTCGCT
CTCTCTCTCTCGAGAGAGAGAGAGAAAAAGT
GTGTCACTAAGTGACACATGCGATGTACATCG
CAAGTCAGTCACAGATCACTTGACATGCCGAT
GAGGGGCAAGCGATCACACCAGCTCGACCAG
TTGTGATCGGTGCAAGCGGGAGCTGGTGGGA
GGGGACGGGTACCTCCCACC

Figure 10 - Illustration of Consensus Sequences derived from the multiple alignment of TE sequences and the *Eucalyptus* spp genome, generated by Jalview.

Using the conservation sequence profiles, a distinct sequence signature was identified for each region, which showed that the areas that encompassed the shared β-hairpin motif were highly conserved. To enhance the visual representation of the marked conservation in these regions, a weblogo was generated (Crooks et al., 2004), which shows the amino acid distribution in colors at each position for each signature motif (Figure 11).

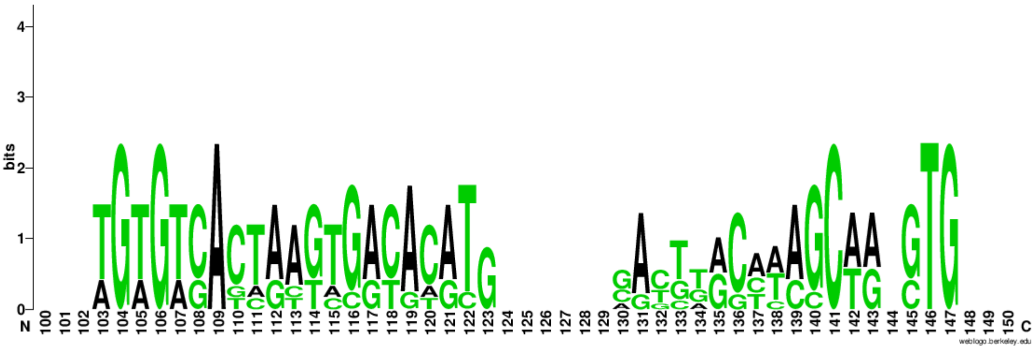


Figure 11 - Sequence signatures based on transposons of aromatic (W, F, Y; in green) and aliphatic amino acids (A, V, L, I, M; in black) in *Eucalyptus* spp.

The analysis of transposon sequences revealed moderate conservation, with varying levels of nucleotide height that indicated their conservation levels. The Weblogo analysis focused on *Eucalyptus* transposases that consisted of 150 amino acids. Aromatic amino acids, in particular tryptophan (W), phenylalanine (F) and tyrosine (Y), have been identified in *Eucalyptus* species and are of interest due to their potential role in insect resistance.

Research has shown that elevated levels of these aromatic amino acids contribute to enhanced resistance against a wide range of insect pests. These amino acids are involved in the synthesis of secondary metabolites, such as phenolics and terpenes, known for their insecticidal properties. Studies have explored the use of genetic manipulation techniques to enhance the expression of the phenylalanine ammonia-lyase (PAL) gene, with a view to increase phenolic compound production and improve resistance against specific insect pests. Aromatic amino acids in *Eucalyptus* are also associated with indirect defense mechanisms against insect pests, as they contribute to the emission of volatile organic compounds that attract natural enemies of herbivores, such as parasitoids or predators, providing an additional layer of defense (Chaudhari et al., 2021).

The relationship between the presence of secondary compounds and the response of *Eucalyptus* genotypes to *L. invasa* attacks (Dantas 2019; Oates 2015) was confirmed by the results of *in silico* analysis.

5.3.3 Genotyping

The genetic markers DAT, UBC 834, Goofy, UBC809, Pat 1 – QF, Pat 1 – QR, Lxx22R, Cxx2, M2, UBC810, UBC808, CLIRAP1, SSR, URP2R, URP6R, URP16R, URP13R and CLIRAP4 were used to differentiate susceptible from resistant *Eucalyptus* genotypes to gall wasp infestation. Polymorphic patterns were observed in the amplification profiles generated by these ISSR markers, indicating variations among the analyzed genotypes. These variations suggest the presence of different alleles or genetic variants at the targeted loci. The number of fragments produced ranged from 1 to 5, with a mean of 2 fragments per oligonucleotide, resulting in a total of 36 fragments.

Data analysis with GenAlEx provided insights into the percentage of polymorphic *loci* within the studied groups. A lower percentage of polymorphism (30.56% of variation) for susceptible plants was detected. On the other hand, resistant plants had a higher polymorphism level (77.78%). The mean percentage of polymorphic groups was estimated at 54.17%, with a standard error (SE) of 23.61% (Figure 12).

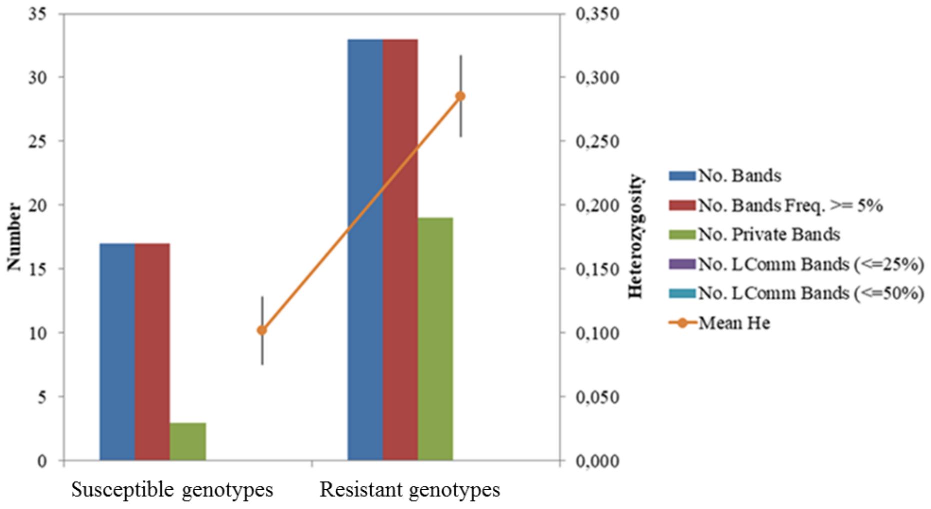


Figure 12 - Band patterns across *Eucalyptus* genotype groups in response to *Leptocybe invasa* based on cumulative data derived from TE and ISSR analysis: Susceptible and Resistant.

Genetic data analysis identified distinct band patterns for the susceptible and resistant genotypes represented in the study, i.e., a total of 17 and 33 bands, respectively, for the susceptible and resistant genotypes.

The presence of private bands, unique to each group, was also examined. Susceptible genotypes had three private bands, indicating specific genetic markers exclusive to this Group. In contrast, resistant genotypes had a higher number of private bands (a total of 19). These private bands contribute to the genetic distinctiveness and uniqueness of each group. The presence of private bands indicates specific genetic differences for DNA regions present in some genotypes but absent in others. Previous studies described the relationship between genetic diversity and resistance to stress or pathogens (Abrinbana et al., 2010; Suzuki et al., 2022).

The analysis further considered the degree of shared bands, known as LComm bands, within and between the group. In this case, neither susceptible nor resistant genotypes had any bands within the categories LComm bands <=25% or <=50%, suggesting few shared genetic markers.

To assess the genetic diversity within each Group, measures such as Mean Expected Heterozygosity (He) and Mean Unbiased Expected Heterozygosity (uHe) were calculated. Susceptible genotypes had a Mean He of 0.102, indicating moderate levels of genetic diversity. The Standard Error (SE) of Mean He of the Groups was 0.027, reflecting the precision of this estimation. Similarly, the Mean uHe calculated for susceptible genotypes was 0.122, with an SE of Mean uHe of 0.032. In contrast, higher levels of genetic diversity were found for the resistant genotypes, with a Mean He of 0.285 and an SE of Mean He of 0.032. The Group Mean uHe was calculated as 0.317, with an SE of Mean uHe of 0.036. These results suggest higher genetic diversity of resistant than susceptible genotypes.

Free Tree software was used to construct a genetic similarity matrix, based on eight samples (1277 S, 1262 S, 1275 S, 1249 R, 1250 R, 1404 R, 0321 R and 5341 R). The analysis detected a range of similarity values among the analyzed samples. The highest similarity (58%) was detected between samples 1277 S and 1275 S. Samples 1275 S and 1277 S were also highly similar to sample 1250 R (50 and 58%, respectively). On the other hand, similarities were lowest (25%) between samples 1249 R and 5341 R. Low similarity was also found between sample 0321 R and samples 1277 S, 1275 S and 1250 R (28, 44 and 26%, respectively).

The dissimilarity data obtained from these primer sets were analyzed to construct trees using TEs and ISSR methods. Transposable Elements and ISSR primers are commonly used in dissimilarity studies due to their ability to detect polymorphisms in different genomic regions (Amiteye, 2021). The dissimilarity values ranged from 20 to 65%. The equality threshold was set to 0%, to ensure that only identical units were grouped together (Figure 13).

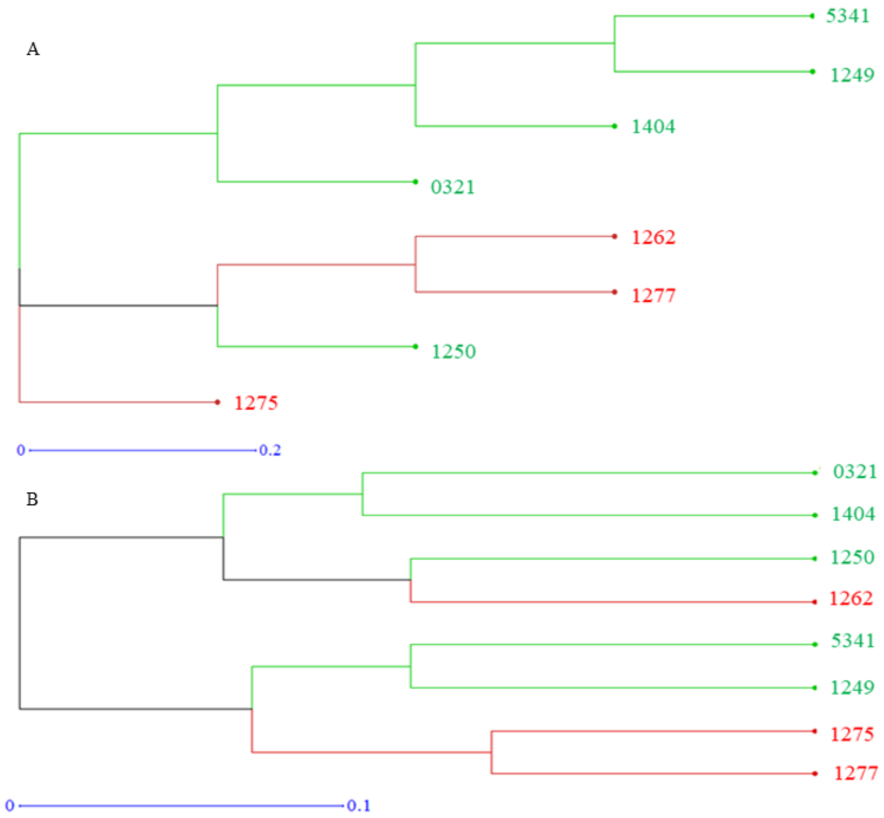


Figure 13 - Dendrogram resulting from UPGMA cluster analysis using Jaccard's dissimilarity coefficient for (A) TE-based markers and (B) ISSR-based markers. Red represents genotypes classified as susceptible, while green represents resistant genotypes.

The dissimilarity data used for tree construction were obtained from the analysis of TE primers (Figure 13-A). The dissimilarity values ranged from 12 to 75%, reflecting the genetic differences among the analyzed units. The tree construction process involved several iterations. The initial iterations grouped genotypes 5341 and 1249 (resistant) together, followed by the grouping of genotypes 1404 and 0321. Subsequent iterations led to the formation of additional groups, ultimately resulting in the last cluster, represented by genotype 1262, 1277, 1250 and 1275.

The edges of the final tree represented the dissimilarity between the connected units. The sum of the edge length was calculated as 1.0, indicating the overall genetic dissimilarity among the units in the tree. The dissimilarity data used for tree construction were obtained from the analysis of ISSR primers (Figure 13-B). In the first iteration, node 9 was formed by grouping (susceptible) genotypes 1277 and 1275. In the second iteration, nodes 10 and 11 were formed by genotypes 5341 and 1249 and units 1262 and 1250, respectively. Subsequent iterations led to the formation of additional nodes, ultimately resulting in the last group represented by node 15, which consists of nodes 14 and 13.The edges and their respective lengths in the final tree were calculated. The edge lengths ranged from 0.042 to 0.14, with a total sum of 1.339.

In this way, the resistance levels among the genotypes 5341, 1249, 1404, 0321, 1277 and 1275 could be differentiated, consistent with the phenotypic analysis.

Molecular markers such as ISSR (Inter Simple Sequence Repeats) provide valuable information on interrelationships among plant genotypes (Azizi et al., 2019; Odesola et al., 2021; Ojuederie et al., 2020).

In view of the potential impact of transposable elements (TEs) on gene regulation, it is important to note that TEs can have diverse effects on neighboring genes, pre-RNA processing and coding sequences (Lee and Rio, 2015). This can influence plant response to stressful situations as that of insect pest attack (Graveley, 2005).

5.4. Conclusions

It was possible to distinguish gall-wasp-susceptible from - resistant genotypes by image analysis.

In silico analysis identified conserved regions in the *Eucalyptus* spp. genome, associated with proteins involved in secondary metabolite production, such as terpenes, which play a role in the plant response to *Leptocybe invasa*.

The discriminatory capacity of TEs and ISSR primers was confirmed and bands were generated and used to identify resistant genotypes. However, in both cases, it is suggested to increase the minimal number of markers used to discriminate genotypes.

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6. FINAL CONSIDERATIONS

This doctoral thesis has made significant contributions to the understanding and management of *Leptocybe invasa* in *Eucalyptus* plantations. By exploring the current state of knowledge on this pest, light has been shed on various management strategies for *L. invasa* control.

This thesis presented academic studies on the feasibility of using biological control, chemical insecticides and resistant genotypes as management practices. The importance of image analysis was emphasized, to facilitate and optimize pest monitoring, providing a practical tool for assessing gall-wasp-induced damage.

Furthermore, the relationship between the quantity of secondary metabolites, particularly terpenes and the response to *L. invasa*, was confirmed by *in silico* analysis. This finding highlights the potential role of these compounds in the defense mechanism of *Eucalyptus* against gall wasp.

For future molecular analyses using transposable elements (TEs) and inter-simple sequence repeat (ISSR) markers the use of a larger number of markers to discriminate *Eucalyptus* genotypes regarding their response to *L. invasa* is suggested. This emphasizes the need for more comprehensive molecular characterization to enhance our understanding of the genetic basis of resistance and facilitate the development of effective control strategies.

In future studies, it is also recommended to further investigate the distribution of the gall wasp, with regard to the impact of global warming. In addition, an in-depth functional analysis of the identified genes and their associated metabolic pathways could deepen the understanding of the mechanisms underlying *Eucalyptus* resistance to *L. invasa*. Knowledge on this topic can contribute to the development of target strategies and the selection of resistant genotypes, to reduce reliance on harmful pesticides and ensure health and productivity of *Eucalyptus* forests in the long term.

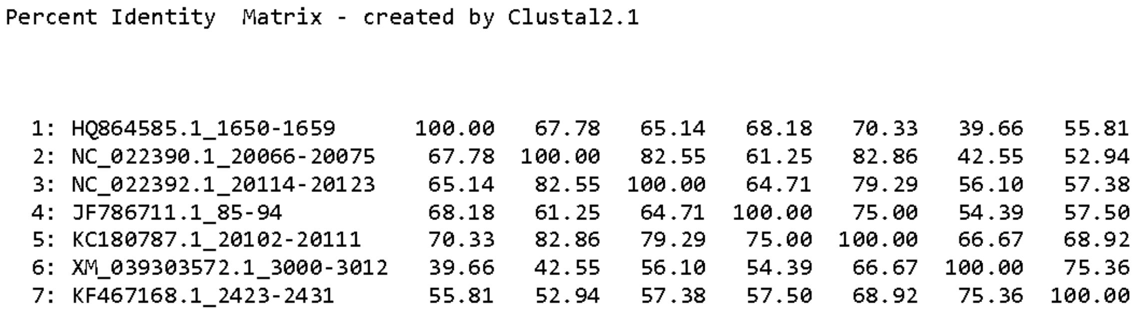
The results of this doctoral research highlight the importance of integrated approaches that combine biological, chemical and genetic strategies for an effective management of *L. invasa* in *Eucalyptus* plantations.

SUPPLEMENTARY MATERIAL

Figure 1S. Multiple Sequence Alignment in Clustal Omega.

```
CLUSTAL O(1.2.4) multiple sequence alignment


HQ864585.1:1650-1659          --------------------CGAGGCCATGCTTTTGACGAGGCCATGCTCGTCAAAGTCT      40
JF786711.1:85-94              -AGCGTGTTCCGCTACAGCGAGGCCATGTCGTCAA-----AGGCCATGCA----TGGCCT      50
KF467168.1:2423-2431          --------------------ACG------------------------GAA----CACCGA      12
XM_039303572.1:3000-3012      ------------------------------------------------TCTGTAGCGTGT      12
KC180787.1:20102-20111        TGTTCCGTACCGCTACAGATACGGAACATGGCCTCGTCGACGAGGCCATTGGCCTCGTCT      60
NC_022390.1:20066-20075       TGTTCCGTACCGCTACAGATACG------------------------GAA----CTGTTC      32
NC_022392.1:20114-20123       TGTTCCGTACCGCTACAGATACGGAACTGTTCCGTGGCCTCGTCATGGCC----TCGCCA      56


HQ864585.1:1650-1659          CTCTCTCTCTCTCTCG------------------TGTGTCACTAAGTGACACACCACCAG      82
JF786711.1:85-94              CTCTCTCTCTCTCTCTCGGT----GTGTCATCACTTGGCAAGTGAGTGACAAAGCTGGTG
106
KF467168.1:2423-2431          GGCCATGTCGTCAAAGAGAGAGAGAGAGAGA-GATGTGTCACTTAA----------GCTG      61
XM_039303572.1:3000-3012      TGCATGGCCTCGTCAGAGAGAGAGAGAGAGAGACTGTTCC--------GTACATGTGGGC      64
KC180787.1:20102-20111        CTCTCTCTCTCTCTCGAGAGAGAGAGAGAGAGAGTGTGTCACTAAGTGACACATGCGATG
120
NC_022390.1:20066-20075       CGTTCT--CTCTCTCGAGAG---------AGAGGTGTGTCACTAAGTGACACATGCGATG      81
NC_022392.1:20114-20123       TGCTCT--CTCTCTCGAGAG---------AGAGGTGTGTCACTAAGTGACACATGCGATG
105
                                      *                         *

HQ864585.1:1650-1659          CTTAGCAACTGATGATCAGA------------------TG------CTGGGCTCCCACCA
118
JF786711.1:85-94              C-----------------------------------------------------------
107
KF467168.1:2423-2431          GT----------------------------------------------------------      63
XM_039303572.1:3000-3012      AAGCTGGTGGATCAGTTGCT----------------------------------------      84
KC180787.1:20102-20111        TAGCTTGCCCACCAAGCTGCGATCAGTTGTGATCGC------------------------
156
NC_022390.1:20066-20075       TACATCGCAAGTGTGTCACGTGTCACTTCACTTGCCGATCAGTTGCAACTGATCAA--CT
139
NC_022392.1:20114-20123       TACATCGCAAGTGTGTCACAGCTTGCCCGGCAAGCTGAAGCTGGTGGGGCAAGCTCACCA
165


HQ864585.1:1650-1659          GCTCCACCAGCT------------------------------------------------
130
JF786711.1:85-94              ----GATCAGTTGTGATC-------------------------------GCACCTCCCAC
132
KF467168.1:2423-2431          ---------------------G------GAAGCTGGTGG---------------------      75
XM_039303572.1:3000-3012      ---------------------------GGGAGCTGGTGGGAGGT----------------
101
KC180787.1:20102-20111        ------CTGGT----------G------GGAGGTGGTGGGAG---GCTGGTGCCTCCCA-
190
NC_022390.1:20066-20075       GATCGCCAGCTACTGATC------------------------------------------
157
NC_022392.1:20114-20123       GCTCGATCAGTTGTGGGAGGTACAAGCTGGTGCTGGTGGGATGGGAGGTATACCTCCCA-
224
```

Figure 2S. Percent Identity Matrix Generated by Clustal Omega



```
 Percent Identity  Matrix - created by Clustal2.1



   1: HQ864585.1_1650-1659       100.00   67.78   65.14   68.18   70.33   39.66   55.81
   2: NC_022390.1_20066-20075    67.78  100.00   82.55   61.25   82.86   42.55   52.94
   3: NC_022392.1_20114-20123    65.14   82.55  100.00   64.71   79.29   56.10   57.38
   4: JF786711.1_85-94           68.18   61.25   64.71  100.00   75.00   54.39   57.50
   5: KC180787.1_20102-20111     70.33   82.86   79.29   75.00  100.00   66.67   68.92
   6: XM_039303572.1_3000-3012   39.66   42.55   56.10   54.39   66.67  100.00   75.36
   7: KF467168.1_2423-2431       55.81   52.94   57.38   57.50   68.92   75.36  100.00
```

Figure 3S. Consensus Sequence Visualization in Jalview