HYBRID WHEAT PRODUCTION: CHARACTERIZATION OF A CYTOPLASMIC MALE STERILITY AND FERTILITY RESTORATION SYSTEM BASED ON *HORDEUM CHILENSE* CHROMOSOME 6 INTROGRESSION INTO THE *TRITICUM DURUM* GENOME

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BIOTECHNOLOGY

BACHELOR'S THESIS

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Tarragona, June 2025

Data de convocatòria: Juny 2025

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FUNDING AND ACKNOWLEDGEMENTS

I would like to sincerely thank everyone who supported me throughout this journey.

First, I want to thank Dr. Azahara C. Martín Ramírez for her guidance and support throughout this work, carried out at the Department of Genetic Improvement and Reproductive Biology of Cereals, Institute of Sustainable Agriculture (IAS-CSIC). This research was made possible thanks to the JAE-INTRO ICU 2024 Fellowship.

I'm especially grateful to Dr. Juan Bautista Fernández Larrea for his guidance, patience, and support, which have been key to the completion of this project.

To the amazing team at the IAS in Córdoba, thank you for welcoming me so warmly, for all the help in the lab, and for making the experience both meaningful and enjoyable.

To Ferran, thank you for being by my side through all of it. Your support, understanding, and belief in me have meant more than words can say.

To my dad and my sister, thank you for being my cheerleaders and for always being there when I needed you.

And finally, I want to thank myself for staying committed, even when things got tough, for showing up every day, and for making it to the finish line.

ABSTRACT

As global food demand rises, sustainable strategies for increasing crop yields are required. Hybrid breeding, which exploits heterosis, offers a promising solution to boost productivity. While hybrid systems are well established in maize and rice, their development in wheat has lagged due to the crop's polyploid genome and self-pollinating nature. Cytoplasmic male sterility (CMS) systems have been proposed as a strategy to force outcrossing in wheat by combining the cytoplasm of one species with the nuclear genome of another, often resulting in mitochondrial-induced sterility. Fertility can then be restored by introducing compatible restorer-of-fertility (Rf) genes into the nuclear genome.

A CMS system known as msH1 was previously developed in bread wheat (*Triticum aestivum*, AABBDD), using the cytoplasm of *Hordeum chilense*. In this system, fertility is restored by a chromosomal translocation that fuses the short arm of wild barley *H. chilense* chromosome 6 ($6H^{ch}S$) with the long arm of wheat chromosome 6D (6DL). However, this system could not be applied to durum wheat (*Triticum turgidum*, AABB) due to the absence of the D genome. To overcome this limitation, this study investigated an alternative translocation, $T6H^{ch}S{\cdot}6AL$, in which $6H^{ch}S$ is fused to the long arm of durum wheat chromosome 6A (6AL), as a potential Rf-carrying line for fertility restoration.

To evaluate this strategy, a durum wheat line carrying the $T6H^{ch}S{\cdot}6AL$ translocation was analyzed alongside the alloplasmic CMS line (H1)TC2.24, which combines *H. chilense* cytoplasm with the durum nuclear genome. To this end, four objectives were addressed: (1) optimize a cytogenetic protocol for chromosome spread preparation, (2) detect and validate the $T6H^{ch}S{\cdot}6AL$ translocation using molecular markers and genomic *in situ* hybridization (GISH), (3) confirm chromosome number and stability in the CMS line, and (4) attempt crosses between the lines to evaluate fertility restoration.

The optimized protocol for chromosome spread preparation (2 hours of N_2O at 11 bars, followed by 1.5 hours of enzymatic digestion) yielded high-quality chromosome spreads. Molecular markers showed that around 30% of 41 restorer plants were homozygous for the translocation. GISH confirmed stable introgression of the $6H^{ch}S$ arm into chromosome 6A. Chromosome counts identified two stable (H1)TC2.24 plants with 28 chromosomes. However, crosses were unsuccessful due to mildew infection and poor flowering synchrony, leaving fertility restoration untested.

Despite this limitation, the successful characterization and validation of the $T6H^{ch}S{\cdot}6AL$ line is a key step toward establishing a functional CMS–Rf system in durum wheat. These findings provide a foundation for future research into hybrid wheat breeding using *H. chilense* introgressions.

KEY WORDS: durum wheat *Triticum durum*, wild barley *Hordeum chilense*, hybrid wheat, heterosis, cytoplasmic male sterility, fertility restoration, chromosomal translocation.

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LIST OF ABBREVIATIONS

Ae.	*Aegilops* (genus of wild grasses related to wheat)
bp	Base pair
CMS	Cytoplasmic Male Sterility
DAPI	4',6-diamidino-2-phenylindole
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
F_1	First filial generation (first-generation hybrid)
GISH	Genomic *In Situ* Hybridization
H. chilense	*Hordeum chilense*
N_2O	Nitrous Oxide
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription PCR
Rf	Restorer-of-fertility
RNA	Ribonucleic Acid
RNA-Seq	RNA sequencing
T. aestivum	*Triticum aestivum*
T. turgidum	*Triticum turgidum*
6H^{ch}S	Short arm of chromosome 6 from *Hordeum chilense*
6AL	Long arm of chromosome 6A from *Triticum turgidum*
6DL	Long arm of chromosome 6D from *Triticum aestivum*
T6H^{ch}S·6AL	Translocation of 6H^{ch}S onto 6AL
T6H^{ch}S·6DL	Translocation of 6H^{ch}S onto 6DL

1. INTRODUCTION

Currently, 150 crops are used to feed most of the global population, with just 12 of these crops providing more than 80% of the energy in our diets. Among these, wheat, rice, and maize are especially significant. They occupy three-quarters of global cereal acreage and are the leading dietary source of energy, carbohydrates, plant proteins and micronutrients worldwide (Loskutov, 2021; Poole et al., 2021). While wheat is the most significant in terms of cultivated area, maize and rice now surpass it in total production (Poole et al., 2021; Poutanen et al., 2022).

The dominance of these three cereals in world agriculture is a reminder of their importance for food security and nutrition. Efforts to improve their yield and resilience to climate change are key for sustaining the world's growing population (Cai et al., 2024; Wiebe et al., 2021). Among these crops, wheat stands out not only for its global importance but also because improving its productivity is particularly difficult.

Looking ahead, the OECD-FAO Agricultural Outlook 2024-2033, a collaborative effort of the Organisation for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization (FAO) of the United Nations, projects a steady rise in demand for staple crops, particularly in low- and middle-income countries. For wheat, meeting this demand will largely depend on improving productivity, as further expansion of cultivated land is limited by environmental and resource constraints (OECD-FAO Agricultural Outlook 2024-2033). This highlights the need for innovative approaches that can sustainably increase yield. In this context, the development of hybrid wheat systems is a potential strategy to achieve both these production and sustainability goals (Gupta et al., 2019; Longin et al., 2012).

1.1. The Need for Hybrid Wheat

One of the most effective strategies for improving crop performance is the development of hybrid varieties, which exploit a genetic phenomenon known as heterosis (hybrid vigor). Heterosis occurs when offspring from two genetically distinct parents present superior traits, such as higher yield, improved resilience to environmental stress or better disease resistance (Gupta et al., 2019). Hybrid maize and rice have been successfully adopted worldwide. However, hybrid wheat (*Triticum spp.*) remains largely underdeveloped due to the absence of an efficient hybrid seed production system (Longin et al., 2012; Loskutov, 2021). This limitation mainly stems from the genomic complexity associated with its polyploid nature and the reproductive biology of wheat, which heavily favors self-pollination (International Wheat Genome Sequencing Consortium (IWGSC), 2014; Singh et al., 2021).

1.2. **Wheat genome**

Unlike diploid crops such as rice and maize, wheat is a polyploid, meaning it possesses multiple sets of chromosomes derived from different ancestral species. Bread wheat (*Triticum aestivum*), the most widely cultivated wheat species, is an allohexaploid with a genomic constitution of AABBDD, totaling 42 chromosomes. On the other hand, durum wheat (*Triticum turgidum* ssp. *durum*), used primarily for pasta, is an allotetraploid (AABB) with 28 chromosomes, lacking the D subgenome (Marcussen et al., 2014).

The evolution of wheat's complex genome began with the hybridization of *Triticum urartu* (providing the A genome) and a species related to *Aegilops speltoides* (providing the B genome), resulting in the formation of wild emmer wheat, a tetraploid ancestor of modern durum wheat. Subsequently, a second hybridization event occurred when domesticated tetraploid wheat hybridized with *Aegilops tauschii*, introducing the D genome and giving rise to hexaploid bread wheat. This polyploidization process allowed wheat to combine genetic material from three distinct ancestral species, greatly expanding its genetic diversity and adaptability (**Figure 1**) (International Wheat Genome Sequencing Consortium (IWGSC), 2014; Marcussen et al., 2014).

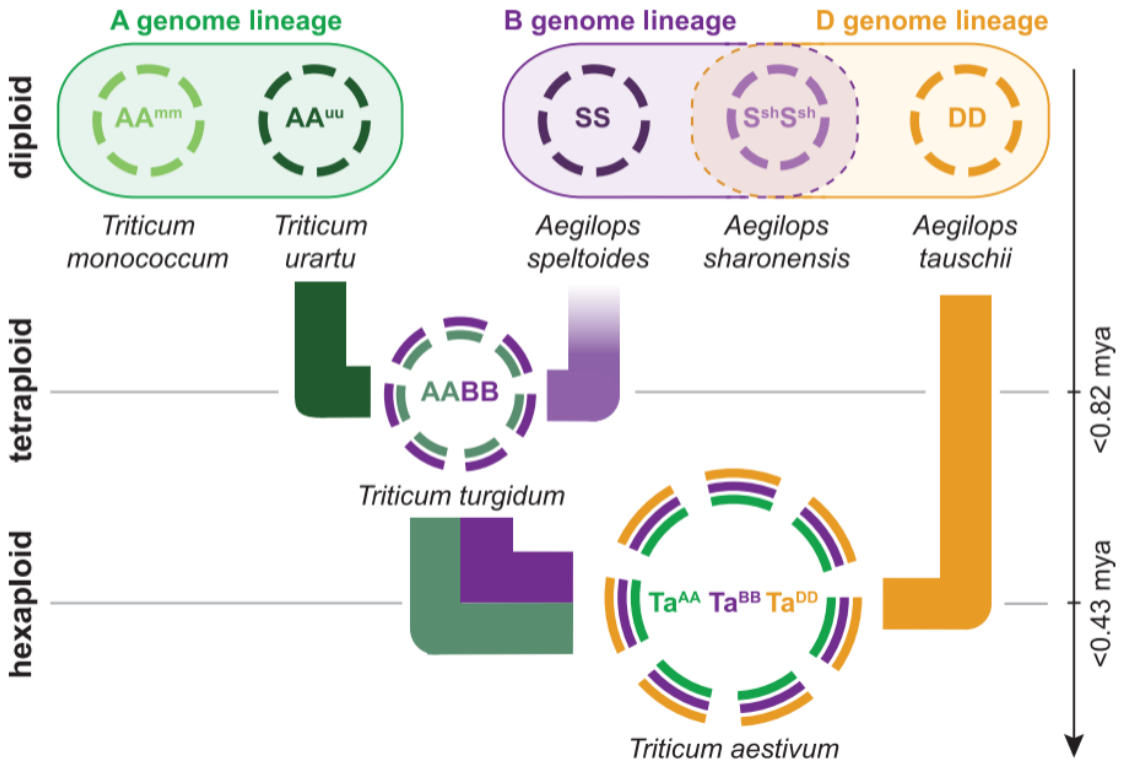

Figure 1. Wheat genome evolution through polyploidization. *Diploid species from the A, B, and D genome lineages contributed to the formation of tetraploid T. turgidum (AABB) and hexaploid T. aestivum (AABBDD) via two successive hybridization events. Time estimates (in million years ago, mya). Figure extracted from Lukaszewski et al., 2014*

Polyploidy, while conferring advantages such as genetic redundancy, greater adaptability, and enhanced stress tolerance, also introduces significant complications for plant breeding. The presence of homoeologous chromosomes (similar chromosomes from different subgenomes) can disrupt regular meiotic pairing, particularly in hybrid lines where genomic balance is perturbed (Nieto Feliner et al., 2020). This disruption often leads to irregular chromosome segregation, aneuploidy, and reduced fertility, all of which negatively impact the genetic stability and agronomic performance of hybrids. For example, the *Ph1* (Pairing homoeologous 1) locus in wheat suppresses recombination between non-homologous chromosomes (Griffiths et al., 2006). While this suppression is essential for maintaining genomic integrity during meiosis, it also creates a barrier to genetic exchange between subgenomes or with wild relatives, which are often used in breeding programs to introduce new traits (Longin et al., 2012; Nieto Feliner et al., 2020).

The size of the wheat genome adds to its complexity. At approximately 17 gigabases, it is one of the largest among cultivated crops, with over 80% consisting of repetitive DNA elements (International Wheat Genome Sequencing Consortium (IWGSC), 2014). This large and repetitive nature of the genome makes sequencing, gene annotation, and molecular marker development particularly challenging. Until recently, the lack of a fully annotated genome has been a significant obstacle. The release of the first high-quality, fully annotated wheat genome in 2018 was a major breakthrough, providing a comprehensive resource for genomic studies and breeding efforts (Appels et al., 2018). This reference genome, along with subsequent assemblies of additional wheat accessions, has helped identify key genes related to fertility, stress response, and yield.

Nevertheless, the redundancy of genes across the A, B, and D genomes complicates functional analysis and the transfer of traits through conventional breeding or genetic engineering. The existence of multiple homoeologous genes can mask the effects of individual gene mutations, making it difficult to establish clear links between specific genotypes and phenotypes. As a result, both the evolutionary success and the breeding challenges of wheat are intimately tied to its polyploid genome (Appels et al., 2018; Melonek & Small, 2022).

1.3. **Wheat's Reproductive Biology and Self-Pollination**

Beyond wheat’s genome complexity, its reproductive biology also hinders hybrid development. As a predominantly self-pollinating species, wheat has evolved floral characteristics that promote self-fertilization and limit cross-pollination, precisely the opposite of what is required for efficient hybrid seed production (Gupta et al., 2019; Sade & Doğan, 2024). Wheat flowers, known as florets, are arranged in spikelets, collectively forming the wheat ear or spike. Each floret contains both male (stamens) and female (pistil) reproductive organs, making them hermaphroditic. The wheat floret

typically contains three stamens with anthers that produce pollen and a single pistil with a bifid feathery stigma receptive to pollen. The anthers and stigma are enclosed within protective structures called the lemma and palea, which limit exposure to external pollen (**Figure 2**) (Okada et al., 2018; Selva et al., 2020).

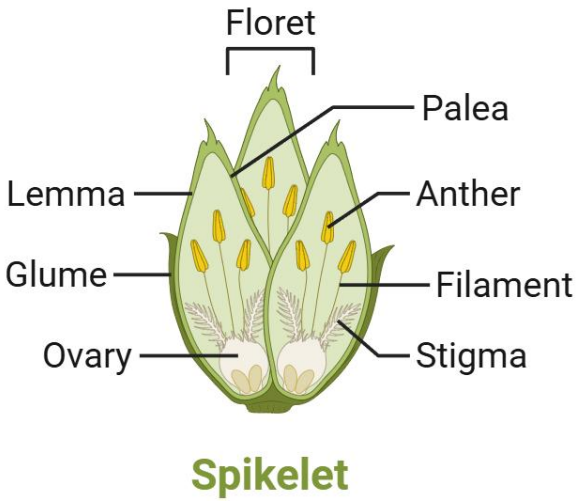

Figure 2. Diagram of a spikelet*, showing key floral structures including the glume, lemma, palea, anther, filament, stigma, and ovary within the floret. Created with BioRender.com*

This floral architecture strongly favours self-pollination, as in most wheat varieties, pollen is released within the closed floret and fertilization happens before the flowers even open (Selva et al., 2020). As a result, opportunities for cross-pollination are extremely limited, even when different varieties are grown nearby. Furthermore, wheat produces limited amounts of pollen, which remains viable for only 30 minutes to a few hours, depending on environmental conditions (Schmidt et al., 2025). The pollen is also relatively heavy and not easily dispersed by wind over long distances, further reducing the likelihood of natural outcrossing (Schmidt et al., 2025).

Several strategies have been explored to increase outcrossing. These include selecting varieties with more open floral structures, applying growth regulators to adjust the timing of flowering, or optimizing field design to increase pollen flow (Gupta et al., 2019; Okada et al., 2018; Selva et al., 2020). However, these approaches have yielded only modest gains. To overcome this problem, one of the most promising strategies has been the use of male sterility systems (Bohra et al., 2016).

1.4. **Male Sterility Systems in Hybrid Wheat Production**

Male sterility systems are required to establish a reliable method to force outcrossing. It is dependent on blocking self-pollination by inducing male sterility or self-incompatibility (Whitford et al., 2013). Several methods have been described, one of them being CMS.

CMS is a condition in plants where an organism is unable to produce functional pollen, resulting in total or partial male sterility (Brownfield, 2021; Melonek et al., 2021). This trait is maternally inherited,

meaning it is passed down through the mitochondrial genome rather than the nuclear genome (Bohra et al., 2016; Melonek et al., 2021).

CMS arises due to specific interactions between nuclear and mitochondrial (or sometimes plastid) genes, often involving mutations or rearrangements in the mitochondrial DNA that disrupt normal pollen or anther development (Melonek et al., 2021; Whitford et al., 2013). While the female reproductive function is unaffected, the inability to produce viable pollen means these plants cannot contribute genetically through male gametes.

A key feature of CMS is that it is non-Mendelian in inheritance, as it is controlled by extranuclear genes and thus inherited only from the mother line (Whitford et al., 2013). In many cases, nuclear-encoded Rf genes can counteract the effects of CMS, restoring male fertility by suppressing the sterility-inducing mitochondrial genes (Castillo et al., 2014; Melonek & Small, 2022).

CMS is widely utilized in agriculture, especially for hybrid seed production, as it enables breeders to produce hybrids without the need for manual emasculation, thus facilitating cross-pollination and harnessing hybrid vigor (Whitford et al., 2013).

One of the most common CMS methods is the three-line system. This approach involves three types of lines: the CMS line (A-line), the maintainer line (B-line), and the restorer line (R-line) (Bohra et al., 2016; Whitford et al., 2013; Xu et al., 2022). The three-line system is schematically presented in **Figure 3**.

The CMS line, also known as the A-line, carries sterility-inducing cytoplasm, often from a different species, and a nuclear genome that does not have genes to restore fertility. Because of this combination, the A-line is male sterile and does not produce viable pollen. This characteristic makes it useful as the female parent in hybrid seed production, as it cannot self-pollinate or pollinate other plants (Bohra et al., 2016; Priyadarshan, 2019).

The maintainer line, or B-line, has a nuclear genome that matches the A-line but possesses normal, fertile cytoplasm. It does not contain fertility-restoring genes. When breeders cross the A-line with the B-line, the resulting seeds inherit the sterile cytoplasm from the A-line and the nuclear background that lacks restorer genes. This cross allows for the continued production of male-sterile A-line plants for use in hybridization (Bohra et al., 2016; Priyadarshan, 2019; Whitford et al., 2013).

The restorer line, or R-line, is a fertile line that carries specific Rf nuclear genes. When the A-line is crossed with the R-line, the hybrid offspring receive the sterile cytoplasm from the A-line and the Rf genes from the R-line. These restorer genes counteract the sterility effect, so the hybrids are able to produce functional pollen and are fertile .

This three-line system is widely used in crops such as rice (Tang et al., 2017), sunflower (Rieseberg et al., 1994), and maize (Levings, 1990). It allows breeders to produce large quantities of hybrid seeds without manual removal of pollen, making hybrid seed production more efficient and practical.

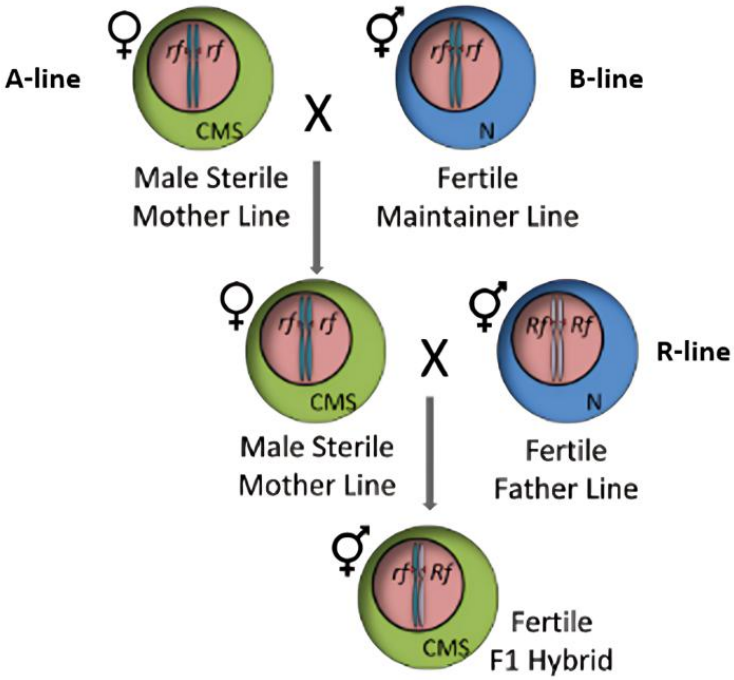

Figure 3. Three-line system used in cytoplasmic male sterility (CMS) for hybrid seed production*. The male-sterile mother line (A-line) carries CMS-inducing cytoplasm and a non-restoring nuclear genotype (rf/rf), making it male sterile. The maintainer line (B-line) has normal cytoplasm and the same nuclear genotype (rf/rf), allowing it to maintain the A-line through backcrossing. The restorer line (R-line) contains normal cytoplasm and at least one restorer allele (Rf), which restores fertility when crossed with the A-line. The resulting F_1 hybrid inherits the CMS cytoplasm from the A-line and a restorer allele from the R-line, resulting in a fertile hybrid suitable for commercial cultivation. Adapted from Whitford et al., 2013.*

1.5. **The msH1 system as a new source of CMS for hybrid production**

While traditional three-line CMS systems have been effective in other crops, wheat has required alternative cytoplasmic sources. The msH1 system in wheat uses the cytoplasm of the wild barley species *Hordeum chilense* to induce male sterility in bread wheat (*Triticum aestivum*) (Martín et al., 2008).

Hordeum chilense is a wild barley native to Chile and Argentina, which shows high potential in wheat breeding due to its high compatibility with *Triticum* species. This allows for the production of fertile and stable amphiploids. An amphiploid is a plant resulting from the hybridization of two different species, followed by chromosome doubling. Therefore, this compatibility facilitates the transfer of *H. chilense* traits to wheat, such as resistance to Septoria tritici (Cifuentes et al., 2024), abiotic stress tolerance (Garthwaite et al., 2005), endosperm storage proteins (Collado-Romero et al., 2015),

carotenoid content (Rey et al., 2015), sterilizing cytoplasms, and Rf genes (Castillo et al., 2014; Martín et al., 2008, 2009, 2010).

The msH1 system is based on nuclear-cytoplasmic incompatibility that arises when the wheat nuclear genome is combined with cytoplasm from the H1 accession of *H. chilense*. The resulting alloplasmic wheat lines are male sterile, meaning they do not produce viable pollen, but otherwise show normal development and fertility on the female side. The sterility is stable across different environments and does not cause major developmental or floral abnormalities, although there may be a slight reduction in plant height and a delay in heading (Martín et al., 2008).

At the cytological level, microsporogenesis in these alloplasmic lines proceeds normally through meiosis, but abnormalities arise during the first mitosis at the uninucleate-pollen stage, leading to failed pollen development and eventual pollen abortion (Martín et al., 2008, 2009).

Fertility restoration in the msH1 system is achieved by introducing specific chromosomal segments from *H. chilense* into the wheat genome. The addition of the short arm of chromosome $6H^{ch}$ ($6H^{ch}S$) from *H. chilense* has been shown to restore male fertility in these alloplasmic lines (Martín et al., 2009). Further studies developed a restorer wheat line, T650, which contains the double translocation $T6H^{ch}S{\cdot}6DL$ (translocation involving the short arm of the *H. chilense* chromosome 6 ($6H^{ch}S$) and the long arm of the wheat D genome chromosome 6 (6DL)) in *H. chilense* cytoplasm, and its ability to restore fertility was confirmed (Martín et al., 2009).

Subsequent research found that the short arm of chromosome $1H^{ch}$ ($1H^{ch}S$) also contributes to fertility restoration, and the combination of both $1H^{ch}S$ and $6H^{ch}S$ provides a more effective restoration of fertility (Castillo et al., 2014; Martín et al., 2010). The restorer effect is linked to the presence of Rf genes located on these chromosome arms, with recent studies identifying candidate genes, including an ortholog of the barley restorer gene Rfm1, in the $6H^{ch}S$ region (Castillo et al., 2014; Martín et al., 2010).

The msH1 system is considered promising for hybrid wheat breeding, as it offers a reliable source of male sterility and a mechanism for fertility restoration. While the msH1 system shows the feasibility of using *H. chilense* cytoplasm for CMS in wheat, ongoing work aims to identify and develop restorer lines that do not negatively affect plant fitness, which is important for practical hybrid wheat production (Castillo et al., 2014).

1.6. **Transferring the msH1 system to durum wheat**

Encouraged by its success in bread wheat, the msH1 system has also been assessed in durum wheat, where comparable results were observed. The substitution of wheat cytoplasm with that of *H. chilense* led to stable male sterility, and the addition of $6H^{ch}S$ restored fertility in some cases (Martín

et al., 2018). Specifically, the system was adapted for use in durum wheat by introducing the cytoplasm of *H. chilense* into the durum wheat nuclear background. This process created alloplasmic durum wheat lines that were completely male sterile, providing a potential platform for hybrid seed production in this crop. Furthermore, to restore fertility, the T6H^{ch}S·6DL translocation was transferred into the durum wheat genome (Martín et al., 2018).

However, using the T6H^{ch}S·6DL translocation in durum wheat presented significant limitations. Durum wheat lacks the D subgenome, so introducing a 6DL-based translocation is not naturally compatible with its AABB genome. In practice, the introduction of T6H^{ch}S·6DL into durum wheat resulted in plants with reduced viability and fertility, especially in the homozygous condition. Many plants carrying the translocation failed to reach maturity, and those that did often showed poor pollen viability or were completely sterile (Martín et al., 2018). The negative effects of the translocation were more pronounced in durum wheat than in common wheat, likely due to the genomic incompatibility and the physiological burden of carrying a large alien chromosomal segment on a background that lacks the D genome (Martín et al., 2018).

Because of these challenges, the T6H^{ch}S·6DL translocation is not optimal for use in durum wheat. Several alternative strategies should be explored, such as transferring the 6H^{ch}S segment to the A or B genome chromosomes (e.g., T6H^{ch}S·6AL or T6H^{ch}S·6BL translocations), or introducing smaller introgressions of 6H^{ch}S, to improve plant fitness and fertility restoration in durum wheat (Martín et al., 2018).

In this work, the T6H^{ch}S·6AL translocation will be explored as an Rf candidate, as it avoids the incompatibility associated with the D genome and may provide a more stable and effective restorer system for hybrid durum wheat breeding.

In summary, while the msH1 system can be transferred to durum wheat, the use of the T6H^{ch}S·6DL translocation is limited by genomic incompatibility and negative effects on plant viability and fertility. Alternative translocations, such as T6H^{ch}S·6AL, will be investigated to overcome these limitations and make the CMS-Rf system more practical for hybrid durum wheat production.

2. HYPOTHESIS AND OBJECTIVES

This study aims to evaluate whether the T6H^{ch}S·6AL chromosomal translocation from *Hordeum chilense* can serve as a candidate for restoring male fertility in cytoplasmic male sterile durum wheat (*Triticum turgidum*). The working hypothesis is that this translocation, when introduced into the nuclear genome of durum wheat, carries Rf genes that counteract the sterility induced by *Hordeum chilense* cytoplasm.

The main goal is to characterize the T6H^{ch}S·6AL translocation line as a potential fertility restorer in an alloplasmic CMS system, using molecular, cytogenetic, and reproductive analyses. By assessing its presence, stability, and interaction with the CMS background, this study seeks to contribute to the development of a functional CMS-Rf system in durum wheat.

The specific objectives of this work are:

1. To optimize a cytogenetic protocol using nitrous oxide (N_2O) treatment to prepare high-quality metaphase chromosome spreads suitable for genomic *in situ* hybridization (GISH) analysis in durum wheat.
2. To validate the presence and homozygosity of the T6H^{ch}S·6AL translocation in selected durum wheat lines using a combination of molecular markers and GISH.
3. To confirm the stability and sterility of the alloplasmic CMS line (H1)TC2.24, evaluating its potential as a reliable female parent in hybrid seed production.
4. To perform controlled crosses between CMS and translocation lines as an initial indication of compatibility and potential fertility restoration.

3. MATERIALS AND METHODS

To evaluate the presence of the T6H^{ch}S·6AL translocation in durum wheat CMS lines, a series of experimental steps were carried out. An overview of the experimental workflow is presented in **Figure 4**. This section outlines the plant materials used, including the CMS line, restorer line, and control lines, followed by a detailed description of the experimental protocols, including N_2O treatment for chromosome spread preparation, molecular marker analysis, and GISH for confirming the translocation.

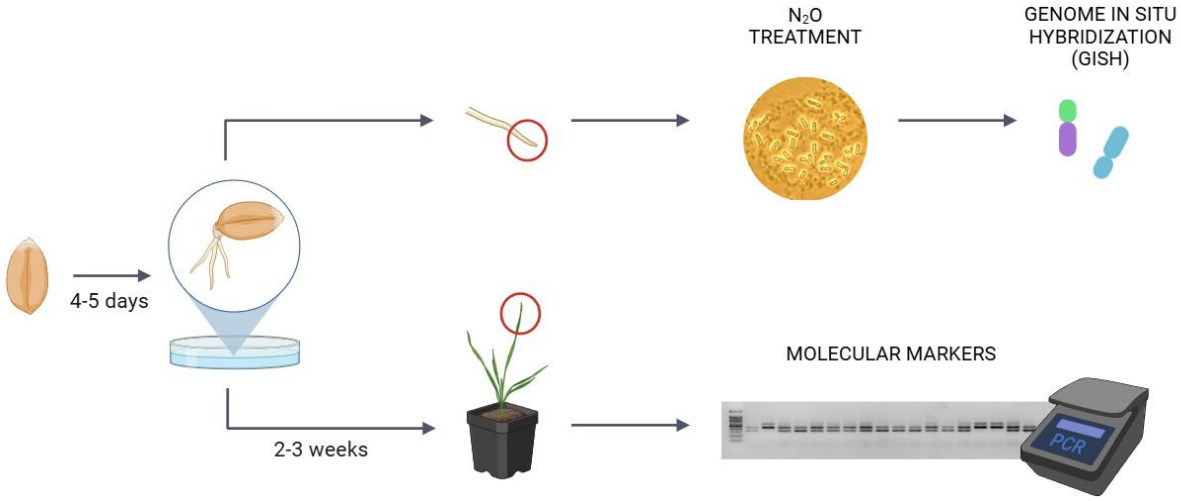

Figure 4. Overview of the workflow used for cytogenetic and molecular characterization in wheat hybrids. *Seeds are germinated for 4–5 days, and root tips are collected for nitrous oxide (N_2O) treatment to arrest cells in metaphase. Genomic in situ hybridization (GISH) is then performed to visualize parental genomes. In parallel, seedlings are grown for 2–3 weeks, and leaf tissue is collected for molecular marker analysis using PCR. This dual approach allows both chromosomal and genetic confirmation of hybrid status. Created with BioRender.com*

3.1. Plant material

The plant material used in this study is described in **Table 1**. The main experimental line, T870, is a *Triticum turgidum* L. var. *durum* line hypothesized to carry the T6H^{ch}S·6AL translocation from *Hordeum chilense* after several generations of self-pollination. This line was the primary subject for evaluating the presence, stability, and potential functionality of the translocation.

The primary CMS line (H1)TC2.24 was developed by crossing the durum wheat variety Don Pedro with a *Hordeum chilense* accession H1 cytoplasmic donor (*Tritordeum*, AABBDDHH). This line exhibits male sterility due to mitochondrial–nuclear incompatibility and was used as the female parent in crossing attempts.

Additional lines were included as controls in the molecular marker analyses. This included H1, the *Hordeum chilense* accession used as the cytoplasmic donor; T21, *Triticum aestivum* cv. Chinese Spring (hexaploid wheat) and Kronos, *T. turgidum* L. var. *durum*.

Table 1. Description of the plant material used in this study.

Line	Germplasm	Cytoplasm (a)	nb (b)
H1	*H. chilense* Roem. et Schultz. accession H1	H^{ch} (H1)	14
Kronos	*T. turgidum* L. var. *durum*	W	28
T21	*T. aestivum* cv. Chinese Spring	W	42
T870	*T. turgidum* L. var. *durum* (T6H^{ch}S·6AL?)	W	28
(H1)TC2.24	Alloplasmic CMS line (*T. turgidum* × H1)	H^{ch} (H1)	?

[a] H^{ch}, Hordeum chilense cytoplasm; W, wheat cytoplasm.

[b] *Chromosome number*.

Seeds were germinated in petri dishes with wet filter paper and kept in the dark at 4°C for four days, or until primary roots emerged. Germinated seeds were then transferred to 22°C overnight, and root tips (approximately 1 cm in length) were cut for chromosome arrest treatment. Remaining seedlings were transplanted into growth chambers maintained under a fixed day-light cycle of 12h and a temperature of 20ºC during the day and 14ºC at night.

3.2. **Nitrous oxide treatment for metaphase spreads**

Chromosome spreads were prepared as described in King et al., (2017) and M. D. Rey et al., 2018), with modifications. Root meristems approximately 1 cm in length were excised from germinating seeds of both the CMS and restorer lines and immediately subjected to N_2O treatment. Root tips were placed in an Eppendorf tube containing 10 µL of distilled water and then transferred into an N_2O pressure chamber. Treatment conditions should be optimized for each species; typically, the chamber is pressurized to 10–15 bar and maintained for 1.5 to 3 hours.

Following N_2O treatment, root tips were enzymatically digested in a solution containing 0.1 g pectolyase Y23, 0.4 g cellulase Onozuka R-10, and 9.5 mL of citric buffer (Yakult Pharmaceutical, Tokyo). The composition of the enzyme mixture may vary depending on the species. However, the most important parameter to optimize is the duration of enzymatic digestion, which can range from 30 minutes to 1.5 hours depending on digestion temperature and laboratory conditions.

After digestion, the enzyme solution was carefully removed. The roots were then washed with 70% ethanol, macerated, and subjected to several additional ethanol washes. Subsequently, root tips were

soaked in 100% acetic acid and incubated for 1.5 hours. Chromosome spreads were then prepared and examined under a phase-contrast microscope.

3.3. **Genome In Situ Hybridization (GISH)**

Chromosome preparation and GISH were performed as previously described (Rey et al., 2018b). GISH is a cytogenetic technique that enables the visualization and differentiation of entire parental genomes within hybrid organisms, particularly in diploid and polyploid hybrids. The method involves labeling total genomic DNA from one or more species with fluorochromes, which then hybridize to complementary DNA sequences on chromosomes. This allows for the identification and mapping of parental chromosome sets within the hybrid genome (Brammer et al., 2013; Silva & Souza, 2013).

GISH is especially useful for analysing the genomic composition and organization in hybrids, detecting intergenomic translocations, and distinguishing parental genomes even in cases of high sequence similarity (Piperidis, 2021; Silva & Souza, 2013). A schematic overview of the method is shown in **Figure 5**.

To label wheat's A and B subgenomes using GISH, total genomic DNA from *Triticum urartu* (A genome progenitor) and *Aegilops speltoides* (B genome progenitor) was used as probes. The genomic DNA of *T. urartu* was labeled with digoxigenin-11-dUTP using the DIG-nick translation mix (Sigma, St. Louis, MO, USA), while the genomic DNA of *Ae. speltoides* remained unlabelled. Additionally, the genomic DNA of *Hordeum chilense* was labeled with biotin-16-dUTP using the Biotin-nick translation mix (Sigma, St. Louis, MO, USA).

The labeled probes were hybridized to chromosome spreads prepared from wheat root tips. After hybridization, the digoxigenin-labeled *T. urartu* DNA was detected using anti-digoxigenin-fluorescein Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany), and the biotin-labeled *H. chilense* DNA was detected using streptavidin conjugated to Alexa Fluor 660 (Thermo Fisher Scientific, Epsom, Surrey, UK). Before imaging, all chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Images were captured using a Leica DM5500B microscope equipped with a Hamamatsu ORCA-FLASH4.0 camera and analyzed using Leica LAS X software to confirm the presence of the T6H^{ch}S·6AL translocation.

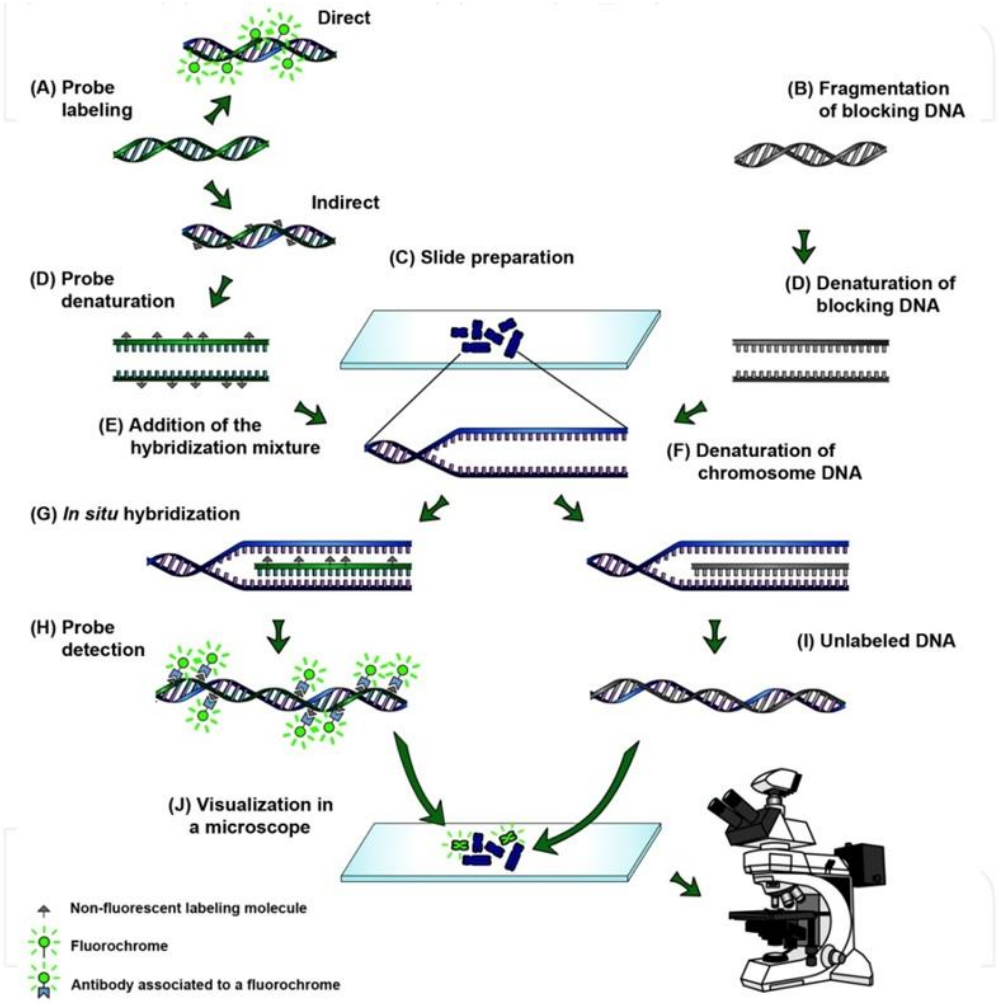

Figure 5. Main steps of the genomic in situ hybridization (GISH) procedure. *(A) Direct and indirect labeling of probes. (B) Fragmentation of the blocking DNA. (C) Preparation of chromosome spreads. (D) Denaturation of the probe and blocking DNA in a hybridization mixture. (E) Application of the hybridization mixture containing the probe and blocking DNA to the slide. (F) Denaturation of chromosomal DNA. (G) In situ hybridization of the probe and blocking DNA to the target chromosome sequences. (H) Detection of the labeled probe in the chromosome DNA of one parent (indirect labeling). (I) Chromosome DNA of the second parent associated with the unlabeled blocking DNA. (J) Visualization of hybridization signals associated with the probe (green) under a fluorescence microscope, with unmarked chromosomes counterstained in blue. In the case of direct labeling, the detection step can be omitted, and the fluorochromes can be visualized directly with the appropriate filter. Extracted from Brammer et al., (2013)*

3.4. **Molecular marker selection**

Leaf tissue was harvested from T870 plantlets for genomic DNA extraction, which was carried out using a modified CTAB protocol (Murray & Thompson, 1980).

Several molecular markers were used to identify specific chromosomal regions. Marker Xgpw3029 was used to amplify the long arm of chromosome 6AL (Rodríguez-Suárez et al., 2020a), while Barc1030 targeted the long arm of 6DL (Song et al., 2005). For the short arm of chromosome $6H^{ch}$, markers HORVU.MOREX.r3.6HG0541800 and HORVU.MOREX.r3.6HG0542120 were utilized

(Rodríguez-Suárez et al., 2020a). In addition, the primer pair FEH_1310-F/FEH_1685-R (Zhang et al., 2008) was used to differentially amplify fragments from chromosomes 6AS, 6BS, and 6DS, enabling the identification of the respective subgenomes.

PCR amplification was conducted using MyTaq™ DNA polymerase (Bioline, London, UK), with an annealing temperature set at 60°C. The PCR products were separated via agarose gel electrophoresis and visualized using SafeView™ Nucleic Acid Stain (NBS Biologicals Ltd, Cambridgeshire, England). Gel images were captured using a Molecular Imager® VersaDoc™ MP Imaging System and analyzed with Quantity One 1-D Analysis Software v4.6.9.

The molecular marker analysis was performed on CMS lines to confirm the successful integration and stability of the T6H^{ch}S·6AL translocation.

3.5. **Chromosome counting**

To verify the cytogenetic stability of the CMS line (H1)TC2.24, Feulgen staining was performed to count the number of chromosomes in wheat root meristem. Plants selected for analysis were preserved in 70% ethanol, subjected to acid hydrolysis with 1N HCl for 11 minutes at 60°C, rinsed with distilled water, and stained with fuchsine for 10 minutes at 25°C in darkness. Chromosome spreads were then observed under the microscope.

4. RESULTS

4.1. Optimization of Nitrous Oxide (N_2O) Treatment for Metaphase Chromosomes Spread Preparation in Wheat

To optimize metaphase chromosome spreads in durum wheat (*Triticum turgidum*) and other wheat lines, three parameters of the N_2O treatment protocol were systematically varied: exposure time, chamber pressure, and duration of enzymatic digestion. Root meristems were treated under different conditions, and the resulting chromosome preparations were evaluated based on the number of complete metaphase spreads per slide, the quality of chromosome condensation and separation, and the level of background debris.

The conditions assessed and their corresponding results are summarized in **Table 2**.

Table 2. Optimization of nitrous oxide (N_2O) treatment parameters for chromosome spread preparation in Triticum turgidum root meristems. *Values represent mean results per slide. * "Complete Metaphases" refers to well-spread, distinguishable metaphase chromosome sets.*

N_2O Exposure (h)	Pressure (bar)	Enzyme Treatment (h)	Complete Metaphases/Slide *	Chromosome Quality	Background Debris
1:30	10	0:45	4	Poor	High
1:30	10	1:00	5	Moderate	High
1:30	10	1:30	6	Moderate	Medium
1:30	11	0:45	6	Moderate	High
1:30	11	1:00	7	Good	Medium
1:30	11	1:30	8	Good	Low
2:00	10	0:45	7	Moderate	High
2:00	10	1:00	9	Good	Medium
2:00	10	1:30	11	Good	Low
2:00	11	0:45	8	Good	Medium
2:00	11	1:00	12	Very Good	Low
2:00	11	1:30	15	Excellent	Very Low
2:00	12	0:45	7	Very Good	High
2:00	12	1:00	8	Good	Medium
2:00	12	1:30	10	Good	Medium
2:00	13	1:00	6	Poor	High
3:00	11	1:00	9	Moderate	Medium
3:00	11	1:30	10	Moderate	Medium
3:00	12	1:30	7	Moderate	Low

Root meristems were exposed to N_2O gas under different conditions of time, pressure, and enzyme digestion to optimize the preparation of metaphase chromosome spreads suitable for cytogenetic analyses. Initially, shorter N_2O exposure times (1:30 h) and lower chamber pressures (10 bar) yielded suboptimal results, characterized by fewer complete metaphase spreads and high background debris. Under these conditions, metaphase chromosomes were visible but poorly condensed, with significant background debris (**Figure 6A**).

Increasing the exposure time to 2 h markedly improved metaphase quality, particularly at a chamber pressure of 11 bar. At a slightly higher pressure of 12 bar and with a digestion time of 1 h, chromosome spreads appeared clearly condensed, although high background debris remained due to insufficient enzymatic digestion (**Figure 6B**).

Different durations of enzymatic digestion (45 min, 1 h, and 1:30 h) at 40 °C were also tested. Digestions of 45 minutes resulted in incomplete cell wall degradation and poor chromosome release (as seen in **Figure 6A**). However, extending digestion to 1:30 h in a solution containing 1% pectolyase and 4% cellulase in 1x citrate buffer significantly improved chromosome morphology and reduced background. The best results were obtained under the combination of 2 h N_2O exposure at 11 bar and 1:30 h enzymatic digestion, which yielded well-spread, clearly condensed metaphase chromosomes with minimal background (**Figure 6C**). On average, 15 complete metaphase spreads per slide were observed under these optimized conditions. These conditions were subsequently used for all cytogenetic analyses in this study.

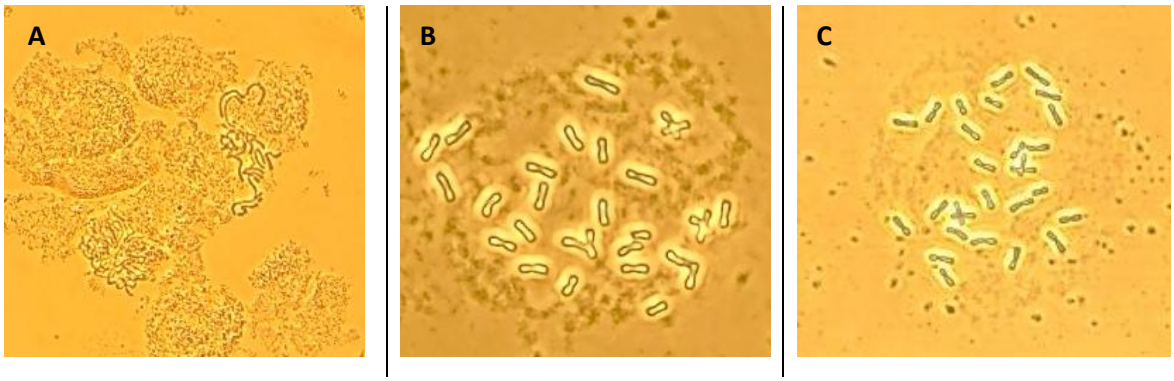

Figure 6. Chromosome spreads *prepared after (A) 1:30 h exposure to N_2O at 10 bar and enzymatic digestion for 45 min, (B) 2:00 h exposure to N_2O at 12 bar and enzymatic digestion for 1:00 h and (C) 2:00 h exposure to N_2O at 11 bar and enzymatic digestion for 1:30 h.*

4.2. Validation and Characterization of the Homozygotic T870 Wheat Line with Double T6H^{ch}S·6AL Translocation in selected durum wheat lines

After optimizing the metaphase spread preparation protocol for cytogenetic analysis, the presence of the T6H^{ch}S·6AL translocation in the durum wheat line T870 was validated. Initial confirmation was performed through molecular marker analysis, targeting *Hordeum chilense*-specific sequences, followed by cytogenetic validation using GISH.

4.2.1. Validation of the T6H^{ch}S·6AL Translocation in T870 Wheat Lines by Molecular Marker Analysis

To confirm the presence of the T6H^{ch}S·6AL translocation from *Hordeum chilense* in the T870 durum wheat line, a panel of molecular markers was used, targeting different chromosomal regions. Specifically, markers HORVU.MOREX.r3.6HG0541800 (P1) and HORVU.MOREX.r3.6HG0542120 (P2) were used to detect the 6H^{ch}S alien chromatin, Xgpw3029 was used to amplify the wheat 6AL arm (indicating the wheat chromosome presence), and FEH and Barc1030 served as wheat endogenous controls for 6AS, 6DS, and 6DL regions.

PCR amplifications were performed on genomic DNA extracted from T870 plants, parental controls (*Triticum durum* Kronos, Chinese Spring (T21), and (H1) *H. chilense*. Amplicons were visualized by agarose gel electrophoresis, and the presence or absence of specific bands was recorded (**Figure 7**). In this figure, the top band in the samples indicates the presence of the alien segment from *H. chilense* in homozygous and heterozygous T870 plants.

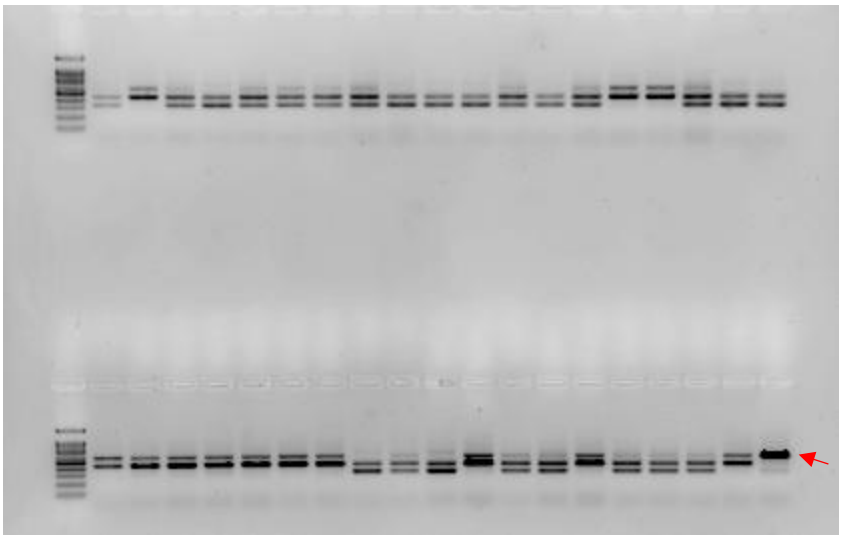

Figure 7. PCR amplification profiles obtained with 6H^{ch}S-specific molecular marker P1 on T870 plants and control lines. *Red arrow indicates the band for 6H^{ch}S amplification.*

The amplification profiles are summarized in **Table 3**, which details the presence (+) or absence (–) of

each marker across the tested plants. Markers P1 and P2 consistently amplified specific bands corresponding to the 6H^{ch}S segment in plants carrying the translocation. Their combined presence, together with amplification of Xgpw3029 (6AL arm), allowed the identification of heterozygous plants (6H^{ch}S/6AL) versus homozygous individuals for either the alien segment or the native wheat 6A chromosome. Control markers (FEH, Barc1030) confirmed the background genomic integrity.

Out of the 41 T870 individuals, approximately 30% (12 plants) were classified as homozygous for the T6H^{ch}S·6AL translocation (presence of alien markers and absence of wheat 6AS markers), 41% (17 plants) as heterozygous (6H^{ch}S/6AL) (presence of both alien and wheat chromatin), and 24% (10 plants) as homozygous for the native 6A wheat chromosome (absence of alien markers, positive Xgpw3029 signal).

Notably, one individual (T870b-1-11) showed absence of amplification for both the wheat 6AL marker Xgpw3029 and the alien markers, suggesting a nullisomic condition for chromosome 6A, likely resulting from chromosomal loss during breeding. Such individuals were excluded from further analyses due to genomic instability.

Additionally, T870-8-1, displayed unexpected amplification at the 6DL marker Barc1030, suggesting possible contamination or rearrangements involving wheat chromosome 6D.

The molecular marker profiles thus confirmed the successful introgression of the 6H^{ch}S·6AL translocation into the T870 wheat background, while also revealing rare chromosomal abnormalities within the population.

Table 3. PCR-based molecular marker analysis to detect the presence of the 6H^{ch}S·6AL translocation in T870 plants and control lines. *Markers P1 and P2 are specific to Hordeum chilense 6H^{ch}S chromatin. FEH was used to discriminate between subgenomes A, B and D. Xgpw3029 and Barc1030 served as internal controls for wheat background.*

	6H^{ch}S	6H^{ch}S	6AS	6AL	6DS	6DL	
Line	P1	P2	FEH	Xgpw3029	FEH	Barc1030	Result
T870-1-1	**+**	**+**	**+**	**+**	**-**	**-**	**Heterozygous 6H^{ch}S/6AL- 6A**
T870-1-2	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870-1-3	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870-1-4	-	-	+	+	-	-	Homozygous 6A
T870-1-5	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6^{a}
T870-1-6	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-1-7	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870-1-8	-	-	+	+	-	-	Homozygous 6A
T870-2-3	-	-	+	+	-	-	Homozygous 6A
T870-2-4	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A

T870-2-5	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870-2-6	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870-5-1	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870-5-3	-	-	+	+	-	-	Homozygous 6A
T870-5-4	-	-	+	+	-	-	Homozygous 6A
T870-5-5	**-**	**-**	**+**	**+**	**-**	**-**	**Homozygous 6A**
T870-5-6	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870-5-7	-	-	+	+	-	-	Homozygous 6A
T870-8-1	**+**	**+**	**+**	**+**	**-**	**+**	**Contamination or 6D**
T870-8-2	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-8-3	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-8-4	**+**	**+**	**+**	**+**	**-**	**-**	**Heterocigota 6HS/6AL- 6A**
T870-8-5	**-**	**-**	**+**	**+**	**-**	**-**	**Homocigota 6A**
T870-8-6	-	-	+	+	-	-	Homozygous 6A
T870-8-7	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-9-1	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-9-2	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-9-3	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-10-1	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-10-2	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870-10-3	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870b-1-1	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870b-1-2	**-**	**-**	**+**	**+**	**-**	**-**	**Homozygous 6A**
T870b-1-3	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870b-1-4	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870b-1-6	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870b-1-7	**+**	**+**	**-**	**+**	**-**	**-**	**Homozygous 6H^{ch}S/6AL**
T870b-1-8	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870b-1-9	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870b-1-10	+	+	+	+	-	-	Heterozygous 6H^{ch}S/6AL- 6A
T870b-1-11	**+**	**+**	**-**	**-**	**-**	**-**	**Chromosome 6A nullisomic**
H1	+	+	-	-	-	-	Control
T21 C.S	-	-	+	+	+	+	
Kronos	-	-	+	+	-	-	

(+ = amplification detected; – = no famplification.)

4.2.2. **Cytogenetic Validation of the T6H^{ch}S·6AL Translocation in T870 Wheat Lines by Genomic *In Situ* Hybridization (GISH)**

Following the molecular validation of the T6H^{ch}S·6AL translocation in T870 plants, cytogenetic confirmation was performed using GISH. This technique allowed the direct visualization of the alien *Hordeum chilense* chromatin integrated into the wheat genome.

GISH analysis was conducted to cytogenetically confirm the presence and chromosomal location of the 6H^{ch}S·6AL translocation in T870 wheat plants. Total genomic DNA from *Hordeum chilense* was used as a probe, while unlabelled wheat DNA served as blocking DNA to suppress background hybridization. Chromosomes were counterstained with DAPI to visualize the entire genome.

In homozygous T870 individuals, two strong fluorescent signals were consistently observed at the distal regions of a homologous chromosome pair, confirming the presence of the 6H^{ch}S arms on both 6A chromosomes (**Figure 8**). Heterozygous individuals showed a single hybridization signal, while negative lines exhibited no detectable labelling of the 6H^{ch}S arm.

The hybridization patterns observed in GISH analyses were fully consistent with the PCR-based molecular marker results. This provided cytogenetic evidence for the stable introgression of the *H. chilense* chromatin into the wheat genome.

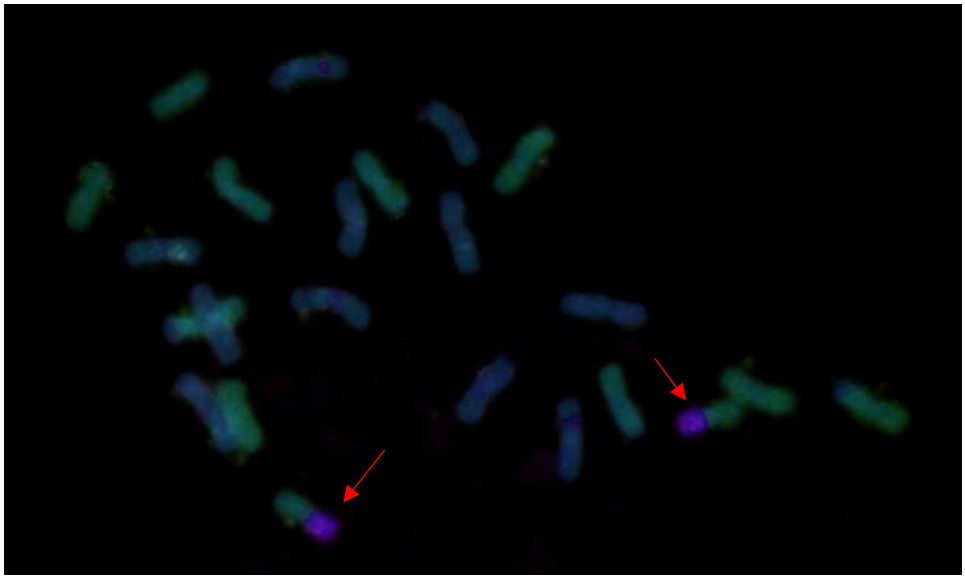

Figure 8. GISH detection of the 6H^{ch}S·6AL translocation in a homozygous T870 wheat plant. *The purple, fluorescent signal (red arrows) corresponds to Hordeum chilense chromatin integrated at the distal end of chromosome 6AL. Triticum urartu genomic DNA probe, which labels the A genome, is shown in green. Blue DAPI staining shows the B chromosomes.*

4.3. **Characterization of the Alloplasmic (H1)TC2.24 Wheat Line for Male Sterility Studies**

After confirming the presence of the T6H^{ch}S·6AL translocation in restorer candidate lines, the next step was to characterize the CMS line (H1)TC2.24, making sure that it had a stable chromosome number (2n=28) appropriate for use as a female parent in hybrid crosses

To confirm the cytogenetic stability of the (H1)TC2.24 line, chromosome counting was performed on root meristem cells using Feulgen staining. Clear metaphase spreads were obtained, allowing accurate assessment of chromosome numbers (**Figure 9**).

Among the four (H1)TC2.24 plants analyzed, two individuals (H1)TC2.24-2 and (H1)TC2.24-3 displayed the expected 28 chromosomes (14 bivalents, corresponding to 7A + 7B chromosome sets of durum wheat). In contrast, (H1)TC2.24-1 and (H1)TC2.24-4 exhibited 28 chromosomes plus additional telocentric fragments, indicative of minor chromosomal instability (Table 4).

The two stable plants with exactly 28 chromosomes were selected for further fertility restoration experiments, ensuring that any observed fertility effects could be reliably attributed to genetic restoration rather than cytogenetic instability.

Table 4. Chromosome counts in (H1)TC2.24 plants following Feulgen staining. *Only individuals with a complete and stable 28-chromosome set were selected for subsequent hybridization experiments.*

Plant Line	Chromosome Number	Observations
(H1)TC2.24-1	28 + telo	Presence of telocentric fragments
(H1)TC2.24-2	28	Normal chromosome number
(H1)TC2.24-3	28	Normal chromosome number
(H1)TC2.24-4	28 + telo	Presence of telocentric fragments

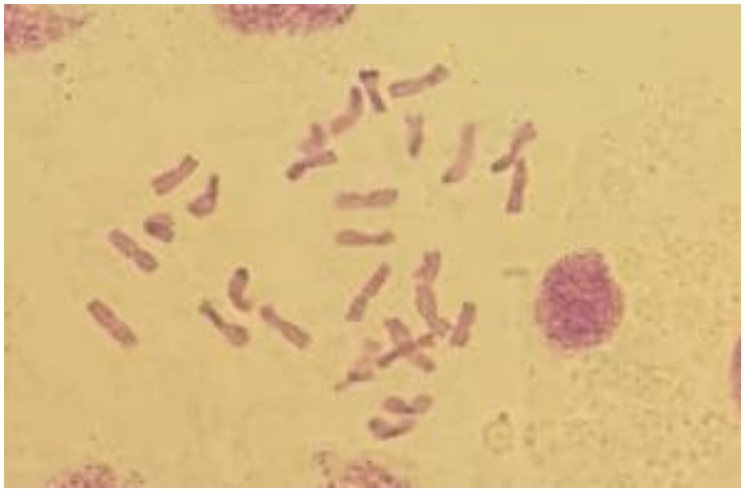

Figure 9. Feulgen-stained metaphase spread from (H1)TC2.24-2 root tip showing 28 chromosomes. (2n = 4x = 28)

4.4. <u>Attempted Crosses Between (H1)TC2.24 and T870 Restorer Lines</u>

After selecting stable (H1)TC2.24 plants and validating T870 restorer candidates, controlled crosses between CMS and restorer lines were planned to assess potential fertility restoration through seed formation.

Controlled crosses between the cytoplasmic male-sterile line (H1)TC2.24 and the T870 restorer candidates were initiated under greenhouse conditions. However, environmental and biological complications prevented the successful synchronization of flowering between the parental lines, and no seeds were obtained during the experimental period.

During the growth period, powdery mildew contamination affected the plants, leading to the application of fungicidal treatments. Although disease control was achieved, treated plants exhibited delayed growth and reduced vigour, likely due to stress responses triggered by both the infection and the chemical treatments. As a consequence, the pollination windows between the CMS line and the restorer candidates became unsynchronized, preventing successful fertilization attempts.

Importantly, due to these complications, it was not possible to determine whether the observed reductions in growth and vigour were solely the result of mildew infection and treatment or whether they were also influenced by the presence of the T6H^{ch}S·6AL translocation.

The crossing experiment will need to be repeated in a future growing cycle, under stricter phytosanitary conditions and better flowering synchronization.

5. DISCUSSION

This study aimed to assess whether the T6H^{ch}S·6AL translocation from *Hordeum chilense* could serve as a potential Rf in a CMS system for durum wheat. The hypothesis was that the 6H^{ch}S arm, previously shown to carry Rf genes in bread wheat, would also be effective when introduced into the A genome of durum wheat. While fertility restoration could not be evaluated due to technical limitations, the results of this study confirm that the 6H^{ch}S·6AL translocation from *Hordeum chilense* can be stably integrated into the durum wheat genome and reliably detected via molecular markers and cytogenetic analysis.

First and foremost, the metaphase chromosome spread preparation using N_2O was optimized. Chromosome visualization plays a central role in cytogenetic studies, and developing a reproducible method that yields high-quality spreads is required. The best results were obtained with a treatment of 2 hours under 11-bar pressure, followed by a 90-minute enzymatic digestion at 40 °C. These conditions consistently produced spreads with a high number of complete metaphases and minimal background debris. This protocol represents a refinement of existing methods. For instance, Kato et al. (2004) first described the chromosome preparation technique in maize, where root tips were treated for 2h with N_2O. However, specific details about the pressure of the nitrous oxide chamber were not given. Later on, King et al. (2017) treated wheat roots with nitrous oxide at 10 bar for 2 h. In this project, it was corroborated that the 2 h treatment grants the best results overall. Nonetheless, a slightly higher pressure (11 bar) granted more consistent results and a higher number of complete metaphases per slide. Furthermore, the enzyme treatment composition was adjusted following recommendations from M. D. Rey et al., (2018) but with modifications to the duration of the enzymatic digestion. Previous studies used 37ºC treatment for 45 to 65 minutes. However, a temperature of 40ºC was used in this study because it is the recommended temperature by the manufacturer (Yakult Pharmaceutical, Tokyo). About the duration, both the 45- and 60-minute digestions caused high background noise that affected the visualization and would make GISH difficult. Here, it was evaluated that a 90-minute digestion provided minimal background.

Importantly, no standardized test was applied to measure reproducibility across environments, so transferability to other labs will require local optimization. Nonetheless, this optimization is consistent with previous work, with modifications suitable for our specific lab conditions (Kato et al., 2004; King et al., 2017; Rey et al., 2018a).

Parallel to cytogenetic optimization, a molecular marker-based screening strategy was used to genotype the T870 durum wheat line. This PRC-based analysis is useful for analyzing a large number of samples in a short time and providing rapid confirmation of the presence of specific genomic segments. In this case, markers for regions of Hordeum chilense, durum wheat and bread wheat were used. In previous work by Rodríguez-Suárez et al., (2020b), markers specific to the Rf H^{ch} chromosomes were found.

Here, two of those markers, HORVU.MOREX.r3.6HG0541800 and HORVU.MOREX.r3.6HG0542120, were selected to target distal and proximal regions of the 6H^{ch}S arm, respectively. The use of both markers ensured that the entire chromosome segment was intact and not subject to terminal deletions, which is critical when attempting to capture the full effect of a candidate Rf region. These markers reliably amplified their targets in all homozygous T870 plants, supporting the presence and stability of the 6H^{ch}S arm.

To discriminate between homozygous and heterozygous carriers of the translocation, a wheat chromosome 6AS marker (FEH_1310-F/FEH_1685-R) was used, which distinguishes between the A, B, and D genome short arms. As expected, homozygous T6H^{ch}S·6AL plants lacked amplification of the 6AS band, indicating complete substitution of the wheat 6A short arm by the *H. chilense* segment. In contrast, heterozygotes retained both wheat and alien bands. Lastly, the integrity of the wheat genome in these lines was assessed using Xgpw3029 and Barc1030, which target 6AL and 6DL, respectively. The consistent amplification of 6AL across most individuals confirmed that no major deletions had occurred. Interestingly, the T870b-1-11 plant tested positive for both *H. chilense* markers but negative for the wheat 6AS and 6AL markers.

This result suggests that the T870b-1-11 plant is in a nullisomic condition for chromosome 6A, where the endogenous wheat chromosome appears to have been entirely replaced by the alien segment. In the context of durum wheat, which lacks a D genome and has limited redundancy compared to hexaploid wheat, such a genotype could result in deleterious phenotypic effects such as reduced viability or fertility due to the absence of essential gene functions typically present on chromosome 6A. This configuration likely arose from a structural rearrangement or segregation error during meiosis and represents a rare but important example of how alien introgressions can destabilize chromosomal integrity (Lv et al., 2023; Przewieslik-Allen et al., 2021). Although no immediate phenotypic defects were observed in this individual, the potential impact on fertility, growth, or gene expression cannot be excluded and warrants further study. Despite these occasional anomalies, the integration of the T6H^{ch}S·6AL segment was stable in most cases.

Following molecular validation, GISH was used to visualize the chromosomal location and configuration of the 6H^{ch}S translocation. Representative plants of each genotype, homozygous, heterozygous, and 6A-only, were analyzed, and the results were fully consistent with marker-based predictions. This dual approach strengthens confidence in the observed results and validates the use of PCR markers as a rapid screening tool for larger breeding populations. The T870b-1-11 plant was not analyzed via GISH due to budget constraints, but slides were preserved for future evaluation.

The cytogenetic analysis of the (H1)TC2.24 line confirmed its stability, with chromosome counts consistently revealing the expected 28 chromosomes and no evidence of additional fragments or structural abnormalities for some plants. This finding is critical, as chromosomal instability in

alloplasmic lines can compromise both fertility restoration studies and hybrid seed production (Schwarzacher et al., 2023). The stable chromosome number in the (H1)TC2.24 line thus supports its potential as a reliable female parent in hybridization experiments, though further testing is required to confirm the absence of minor chromosomal rearrangements that may affect fertility.

Despite successful molecular and cytogenetic validation of the 6H^{ch}S·6AL translocation, the study was unable to assess fertility restoration due to unsuccessful synchronization of flowering between the CMS and restorer lines, compounded by mildew infection and fungicide treatment. While the primary focus of this study was the validation of the T6HchS·6AL translocation, the observed reduction in plant vigor and delayed growth in T870 plants during the attempted crosses raises important questions. Although these effects were most likely exacerbated by fungal infection and fungicide exposure, the possibility that the introgressed *H. chilense* chromatin may contribute to reduced fitness cannot be excluded. Previous studies have reported that large alien chromosomal segments can sometimes introduce linkage drag, negatively affecting agronomic performance (Lv et al., 2023; Martín et al., 2008; Przewieslik-Allen et al., 2021). Durum wheat's lower buffering capacity compared to bread wheat makes it especially vulnerable to dosage effects and imbalances introduced by alien segments, even when they are integrated into a compatible genome.

However, it is also notable that the T870b-1-11 plant, which appeared to be nullisomic for chromosome 6A, survived to the seedling stage. This suggests a degree of tolerance for chromosomal imbalance in the early stages of development, though such plants are unlikely to be viable or fertile in the long term.

Although fertility restoration could not be assessed in this study, the presence of the full-length 6H^{ch}S segment, which includes markers near the known Rf region, supports the hypothesis that the T6H^{ch}S·6AL translocation may function analogously to the T6H^{ch}S·6DL system previously characterized in bread wheat (Martín et al., 2009). If so, this translocation would provide a valuable alternative for restoring fertility in durum wheat CMS lines without relying on the D-genome, which is absent in this species. Further experimentation is required to test this potential, including pollen viability assessments, seed set evaluations, and crossing experiments under synchronized conditions.

Nevertheless, previous research has demonstrated that the 6H^{ch}S arm carries Rf genes capable of reversing CMS in the msH1 system (Castillo et al., 2014; Martín et al., 2008). The presence of robust *H. chilense*-specific markers spanning the distal and proximal ends of 6H^{ch}S supports the integrity of the segment in the translocated lines evaluated here. Thus, the lines characterized in this work remain strong candidates for future fertility restoration trials.

5.1. **Future Perspectives**

To transform these findings into a practical hybrid durum wheat system, a multi-step strategy combining cytogenetic, molecular, and agronomic techniques is proposed. The first step involves testing the functional capacity of the T870 restorer lines by crossing them with the (H1)TC2.24 CMS line under greenhouse conditions. This requires strict disease prevention measures and precise timing to synchronize flowering. Pollen viability can be assessed using Alexander staining, and the seed set rate in the resulting hybrids will indicate the effectiveness of fertility restoration. This stage would confirm the utility of the T6H^{ch}S·6AL translocation, similar to its proven success in bread wheat (Martín et al., 2008).

Simultaneously, molecular analysis is needed to understand how Rf genes function in this system. By comparing gene expression profiles in hybrids and sterile controls, we can identify key regulatory pathways. Techniques like qRT-PCR and RNA-Seq will help pinpoint whether fertility restoration is driven by transcriptional changes or occurs post-transcriptionally. Target genes on the 6H^{ch}S segment, such as the barley Rfm1 ortholog (Castillo et al., 2014), are prime candidates for this analysis.

To minimize unwanted genetic baggage from large chromosomal segments, the next step involves refining the Rf locus on 6H^{ch}S. This can be achieved by generating smaller segments through radiation hybrid lines (Przewieslik-Allen et al., 2021) or creating alternative translocations. Other techniques like CRISPR may further streamline these segments, targeting essential genes while discarding unnecessary genetic material. This strategy has been successful in rice CMS systems (Lu et al., 2021).

Going forward, it will be essential to test whether the T6H^{ch}S·6AL translocation restores fertility in alloplasmic durum lines such as (H1)TC2.24. Additional research should also focus on evaluating potential fitness trade-offs, exploring the use of alternative *H. chilense* accessions as cytoplasmic donors, and generating smaller introgressions to minimize linkage drag. If successful, this system could help overcome a key bottleneck in hybrid wheat production.

6. CONCLUSIONS

Given the results obtained in this study, including the optimization of cytogenetic techniques, the molecular and cytogenetic validation of restorer candidates, the characterization of the CMS line, and the preliminary crossing attempts, the following conclusions can be drawn:

1. A 2-hour nitrous oxide (N_2O) treatment at 11 bar followed by 1:30 hours of enzymatic digestion to durum wheat root meristems provides high-quality chromosome spreads, with minimal background noise.

2. Specific PCR-based molecular markers and genomic *in situ* hybridization confirm the stable introgression of the T6H^{ch}S·6AL translocation from *Hordeum chilense* in the T870 wheat line and allow identification of homozygous and heterozygous individuals.

3. The (H1)TC2.24 cytoplasmic male-sterile line is cytogenetically stable, showing the expected 28 chromosomes without extra fragments, validating its use as a female parent for hybridization experiments.

4. Controlled crosses between the (H1)TC2.24 CMS line and T870 restorer candidates were unsuccessful due to mildew infection and fungicide treatment, preventing growth synchronization, flowering, and evaluation of fertility restoration.

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8. SELF-ASSESSMENT REPORT

When I began my bachelor's Thesis and research training, my main expectations were to gain practical experience in plant biotechnology and genetics, and to develop a deeper understanding of the challenges involved in hybrid wheat breeding. I was particularly interested in learning advanced laboratory techniques in molecular biology and cytogenetics, and in contributing to research that could have a real impact on sustainable agriculture. I also hoped to improve my problem-solving skills and to experience first-hand the day-to-day work of a research laboratory.

Throughout this project, I have acquired a wide range of scientific skills. I learned to prepare and analyze metaphase chromosome spreads using nitrous oxide induction, and to perform genomic in situ hybridization (GISH) to identify chromosomal translocations in durum wheat. I also became proficient in DNA extraction, PCR, and the use of molecular markers to confirm the presence of alien chromosomal segments.

Beyond technical skills, I gained valuable experience in experimental design, troubleshooting, and data analysis. I learned to adapt protocols when initial results were not as expected, and to reflect critically on the experimental process. This hands-on experience helped me understand the importance of patience, resilience, and flexibility in scientific research. Working in the laboratory also improved my ability to collaborate within a research team, communicate scientific results, and manage my time effectively.

On a personal level, this experience has been transformative. I have grown more confident in my abilities and in my capacity to work independently. Facing and overcoming challenges in the laboratory taught me to view obstacles as opportunities for learning and to appreciate the iterative nature of scientific progress. I learned that failure is a natural and essential part of research, and that perseverance and adaptability are key to moving forward.

I am particularly proud of having contributed to the development and validation of a new restorer line in durum wheat, even though not all planned crosses could be completed. This taught me to manage frustration and to value partial achievements as important steps in the scientific process.

Overall, this project has exceeded my initial expectations. It has motivated me to seriously consider a future career in research. I leave this experience not only with new knowledge, but also with essential values and attitudes such as perseverance, adaptability, and teamwork.