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A case study of potato germplasm enhancement using distant late blight resistant wild relatives

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Abstract

Improving potato late blight (LB) resistance is essential for ensuring food security, particularly using wild relatives endemic to *Phytophthora infestans*-prone regions. However, reproductive barriers can impede the transfer from potato wild relatives (PWR) to the cultivated gene pool. This study sought to incorporate potentially novel LB resistance from wild diploid accessions of Series Piurana (*Solanum chiquidenum*, *Solanum paucisectum*, and *Solanum piurae*), Tuberosa (*Solanum cajamarquense*), and Megistracroloba (*Solanum sogarandinum*) into tetraploid potato. Through rescue pollination, 699 diploid interspecific hybrids were obtained, of which 385 displayed LB resistance in two endemic environments. Based on a comprehensive evaluation, including assessing 2n pollen production, 14 diploid interspecific hybrids were selected to continue the introgression process. These pre-bred stocks were then used in interploidy (4x-2x) crosses to incorporate their LB resistance in the cultivated tetraploid background. Assessment of 1978 genotypes resulting from interploidy crosses under endemic disease pressure yielded 717 hybrids with moderate to high levels of LB resistance. Evaluation of pollen viability and ploidy revealed moderate fertility and predominantly tetraploid genotypes. Reassessment of LB resistance on this new subset of hybrids further identified 214 genotypes with sustained resistance. Among them, 12 tetraploid hybrids with low glycoalkaloid content, favorable agronomic, and post-harvest attributes were identified as crossing-friendly stocks. Notably, 11 of these hybrids were derived from *S. cajamarquense* and one from *S. sogarandinum*. These promising 4x hybrids are now primed to be incorporated into potato breeding programs.

Abbreviations: BLUPs, best linear unbiased predictions; CIP, International Potato Center; EBN, endosperm balance number; ER, embryo rescue; FW, fresh weight; GP, gene pool; HER, hybrids from crosses with ER; HI, haploid inducer; HR, highly resistant; HS, highly susceptible; LB, late blight; MR, moderately resistant; PWR, potato wild species; rAUDPC, relative area under the disease progress curve; SSR, microsatellite markers.

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1 | INTRODUCTION

Cultivated potato (*Solanum tuberosum* L., $2n = 4x = 48$) is a key food and cash crop grown worldwide (Bradshaw & Ramsay, 2009). However, its production is severely impacted by late blight (LB), a disease caused by the oomycete *Phytophthora infestans*, which can result in significant yield losses,

particularly in low input agricultural systems. Early LB breeding efforts focused on introgressing resistance genes (*Rpi*) from the wild species, *Solanum demissum*, but many of these varieties were eventually overcome by virulent strains of the pathogen, resulting in a heavy reliance on fungicides for disease control. Later, *Rpi* genes were discovered in various potato wild species (PWR), providing potato breeders with promising new sources of resistance.

Recently, Paluchowska et al. (2022) compiled a comprehensive list of previously identified *Rpi* genes, providing valuable information for potato breeders in their efforts to develop resistant varieties. However, traditional breeding, even with molecular markers, to introduce *Rpi* genes into tetraploid elite potato clones or already released varieties is challenging due to the high heterozygosity and other attributes that must be considered in selection. Genetic engineering is a viable option if regulatory systems permit, and various examples of such products have been documented (e.g., Ghislain et al., 2019; for further examples, see Paluchowska et al., 2022). Pyramiding *Rpi* genes aided by marker assisted introgression followed by backcrossing in (nearly) homozygous diploid potato lines is a new promising technology possible after enabling self-compatibility in diploid potato (Su et al., 2020).

The use of several PWR in breeding is hindered by genetically-based biological barriers such as ploidy, endosperm balance number (EBN), and linkage drag (Jansky, 2006). For instance, the EBN hypothesis, which is a postzygotic barrier, has been extensively studied in potatoes. According to this hypothesis, successful fertilization can only take place when the developing endosperm has a 2:1 ratio of maternal to paternal genetic material. Crossability with cultivated potatoes is used to classify PWR, with species in gene pool 1 (GP1) easily and directly crossed with cultivated potatoes, while species in GP2 and GP3 require aid or manipulation for hybridization. As a result, only few LB-resistant PWR, such as *Solanum bulbocastanum*, *Solanum microdonatum*, *Solanum sparsipilum*, *Solanum stoloniferum*, *Solanum venturii*, *Solanum verrucosum*, *Solanum chacoense*, *Solanum capsicibaccatum*, and *Solanum edinense* have been used in the development of potato varieties (Blossei et al., 2021; Keijzer et al., 2022).

The International Potato Center (CIP) actively surveys underutilized PWR to identify novel sources of LB resistance within species that have evolved in LB-prone environments, but not yet been used in breeding. Pérez et al. (2000) evaluated 133 *Solanum* accessions in 51 tuber-bearing species of 13 taxonomic series for LB resistance and identified 16 species containing at least one accession with either moderate or high levels of LB resistance. CIP's breeding program then selected species from Series Piurana (*Solanum chiquidenum*, *Solanum paucissectum*, and *Solanum piurae*), Series Megistracroloba (*Solanum sogarandinum*), and Series Tuberosa (*S. cajamarquense*) for further testing. They evaluated 40 geno-

Core Ideas

- This study sought to incorporate potentially novel late blight (LB) resistance from underutilized wild relatives into tetraploid potato.
- Promising 4x hybrids from distant wild relatives exhibit low glycoalkaloid content and favorable agronomic traits.
- Overcoming interspecific barriers for novel late blight resistance alleles in potatoes was discussed.

types from up to three accessions of each species for LB resistance in two endemic field locations in Peru. Villamon et al. (2005) described the presence of *Rpi* genes in two accessions of *S. paucissectum* (CIP762124 and CIP762126). Concurrently, CIP's LB resistance assessments of accessions of *S. chiquidenum* (CIP761870 and CIP762573) and *S. piurae* (CIP761868) indicated the presence of major *Rpi* genes. Conversely, three accessions of *S. cajamarquense* (CIP762616, CIP762619, and CIP762620) showed a wide range of resistant genotypes (Lindqvist-Kreuzer et al., 2010; Pérez et al., 2000). Ultimately, 56 accessions were chosen as the most LB resistant to be included in further studies.

This paper presents the results of a systematic approach for incorporating potentially novel LB resistance from underutilized PWR into tetraploid potato. Our study describes the rescue pollination, sexual polyploidization, resistance, and fertility assessment applied to develop pre-bred stocks carrying LB resistance from distant wild relatives.

2 | MATERIALS AND METHODS

2.1 | Generation of *Solanum* interspecific hybrids

2.1.1 | Diploid crosses

Pistillate parents were chosen based on good agronomic characteristics and high nutritional content from a pool of 22 diploid landrace cultivars and bred stocks. Staminate parents were selected from 57 diploid wild tuber-bearing accessions belonging to Piurana, Tuberosa, and Megistracroloba Series, with reported LB resistance (Pérez et al., 2000) were selected as staminate parents. (Table S1). The wild species *S. chiquidenum*, *S. paucissectum*, *S. piurae*, *S. cajamarquense*, and *S. sogarandinum* will hereafter be referred to by the acronyms *chq*, *pcs*, *pur*, *cjm*, and *sgr*, respectively.

The pistillate parents were emasculated, and their stigmas were pollinated with the wild parent pollen, also known

as the pioneer pollen. Subsequently, they were pollinated with a mentor pollinator (IvP101), which is also a haploid inducer (HI), on the following day. This technique, referred to as rescue pollination, involves double pollination with a mentor pollen followed by embryo rescue. Previous studies (Ramon & Hanneman, 2002; Singsit & Hanneman, 1990) have described this approach.

2.1.2 | Embryo rescue

This technique was performed as described by Ordoñez et al. (2017). The hybrid progeny resulting from the interspecific crossing were assigned the code “ER” to reference the embryo rescue technique used in their development. Subsequently, all ER hybrids that prospered in the greenhouse were assessed for their resistance to LB, pollen viability, and unreduced gamete production (see following sections) to identify the most promising genotypes for crossing with tetraploid lines.

2.1.3 | Interploidy crosses following evaluation of late blight resistance, agronomic, and reproductive features

Fourteen advanced 4x lines (referred to as Adv Clones henceforth) from CIP’s breeding program (Table S1) were selected to participate in the interploidy (4x-2x) crosses with 14 2x ER hybrids. These ER hybrids were chosen for their LB resistance, high pollen viability, unreduced gamete production, and good agronomic performance. The aim was to develop tetraploid hybrids that incorporated LB resistance from distant wild relatives, with a view toward broadening the genetic base, and thus the durability of the resistance. The resulting progeny from these interploidy (4x-2x) crosses is hereafter called “HER-hybrids” (Hybrids from crosses with ER).

The 2x-2x crosses to generate ER hybrids and the 4x-2x crosses to generate HER hybrids were conducted in 2006–2007 and 2012, respectively. All crosses were performed in greenhouses located at CIP’s experimental station in the Peruvian Andes (3216 masl [meters above sea level], latitude: -12.01039° , longitude: -75.22411°), with an average temperature of 19.5°C during the day and 11.6°C at night, and relative humidity ranging from 56% to 87%. The numbers of pollinations carried out for each hybridization experiment and the resulting number of flowers, fruits, embryos, and seeds were recorded.

2.2 | Ploidy estimation, cytological, molecular and morphological characterization

Greenhouse-grown plants of ER and HER genotypes were subjected to ploidy and pollen viability evaluation. Ploidy

of all ER genotypes was estimated by chloroplast counting at the stomatal level as described by Ordoñez et al. (2017). Pollen viability and frequency of unreduced pollen were assessed by acetocarmine glycerol-jelly staining as described by Ordoñez et al. (2017). Quispe (2011) provided a comprehensive morpho-agronomic characterization and hybridity test on a subset of 165 ER genotypes, which were selected for their moderate to high resistance to LB. Briefly, the diploid parents and ER progeny were evaluated with 12 microsatellite markers (SSR) from the reference set described by Ghislain et al. (2004). Two of these markers were found to be polymorphic between cultivated and wild parents and provided informative data on the hybridity of the respective progeny. Furthermore, the same subset of parental and ER genotypes underwent morphological characterization using 27 descriptors based on Gomez (2006), and their ploidy was confirmed via flow cytometry as detailed by Ordoñez et al. (2017).

2.3 | Late blight resistance assessments of ER and HER hybrids

The LB resistance levels of parents, hybrids, and controls, which were greenhouse-adapted plants from in vitro culture, were carried out in highland tropical environments during rainy seasons under endemic *P. infestans* infection. The field assessments were conducted only on well-established plants. Weather conditions during the field assessments are recorded in Table S2. Protection against LB with a contact fungicide was suspended 42–45 days after transplanting. Weekly recordings were taken of the percentage leaf area with LB symptoms of each plot until the susceptible control reached 100% of infection. Controls used were: CIP701209 (cv. Chata Blanca) as highly susceptible (HS); CIP720064 (cv. Yungay) as susceptible, and CIP387164.4 (LBr-40) as highly resistant (HR). The relative area under the disease progress curve (rAUDPC) was calculated using the method described by Forbes et al. (2014) and Yuen and Forbes (2009).

The ER hybrids were evaluated at two trial sites in Peru: Oxapampa (1810 masl, -10.57745° , -75.4043°) and Monobamba (1489 masl, -11.35683° , -75.32391°) in 2007 and 2008, respectively. Both field trials followed a randomized block design with three replications and experimental units of four hill-plots. The HER hybrids were initially evaluated in Monobamba during 2012 using an alpha design with two replications of five-hill plots. Later, the HER hybrids that showed moderate to high resistance to LB were evaluated at both Monobamba and Oxapampa trial sites in 2014 using an alpha design with three replications of five-hill plots for each site. Furthermore, both the ER and HER hybrids were grouped based on their wild parent origin.

2.4 | Agronomic assessments of HER hybrids

The agronomic attributes of 355 HER hybrids, which were selected based on their LB-resistant levels ranging from moderately resistant (MR) to HR, were evaluated at the CIP experimental station in La Molina (244 masl, -12.076289° , -76.948417°) during winter 2013. The trial was conducted using an alpha lattice design with two replications of five-hill plots. The HER tubers were harvested in 120 days. Agronomic variables were recorded based on CIP's procedures for standard evaluation of advanced potato clones (De Haan et al., 2014). These variables were as follows: total tuber number per plant, tuber weight (kg) and yield (kg/plant), and number and length of stolons.

Furthermore, glycoalkaloid content assessment was conducted on a subset of 17 HER hybrids. Ten peeled and unpeeled tubers of medium and uniform size were sampled from each HER hybrid and replication. The samples were freeze-dried, milled, and stored at -80°C for analysis, as described by Hellenäs (1986). The percentage of dry matter was also estimated as described by Paget et al. (2014).

A schematic diagram illustrating the workflow of this study is shown in Figure 1.

2.5 | Statistical analysis

The data analysis was performed using R Studio software, version 4.2.3 (R Core Team, 2022). To evaluate the impact of double pollination with IvP101 on rescue pollination, non-parametric test, including Kruskal–Wallis rank sum test, and the chi-square test, were employed as appropriate.

To analyze LB resistance of the hybrids, both ER and HER, a combined analysis approach was used. The analysis was performed as follows: first, the hybrids were grouped based on their wild parent of origin. Then, a linear mixed model was fitted using the ASReml-R package, which considered data where both the hybrids (either ER or HER) and their parents were evaluated in two different environments (e.g., ER hybrids assessed in Oxapampa and Monobamba, in 2007 and 2008, respectively, and HER hybrids assessed in Monobamba and Oxapampa in 2014).

The linear mixed model accounted for fixed effects for environment, experiment, the interaction between environment and experiment, and replications in each experiment, as well as random effects for the genotypes (hybrids and parents) and their interactions with the environments.

The model was able to estimate the LB resistance values for both the hybrids and parents using the best linear unbiased predictions (BLUPs). These values will be referred to as the BLUPs-predicted rAUDPC.

For the purposes of downstream analysis and visualization, progenies were grouped in five categories based on the

BLUPs-predicted rAUDPC values as follows: HR, resistant, MR, moderately susceptible, and HS.

3 | RESULTS

3.1 | Assessment of interspecific reproductive barriers

To facilitate access to the underutilized accessions described in this study, the development of pollen tubes in the pollinated style in crosses between diploid landrace cultivars and bred stocks with wild species from Series Piurana, Megistracroloba, and Tuberosa was examined. Arrested pollen tubes were observed and only few reached the ovules, which restricted fruit formation (Figure 2). The overall degree of interspecific barriers in these crosses was measured using the matrix of pollen tube length and amount described in Trognitz (1991). The presence of both pre- and postzygotic barriers in these crosses was suggested by our observations (data not shown). Hence, rescue pollination was chosen as a means to overcome both types of reproductive barriers in these crosses.

3.2 | Diploid interspecific crosses and rescue pollination

Among the 6514 flowers of diploid cultivated potato pollinated with pollen from selected wild species, a total of 2780 fruits were obtained. Of these, 857 fruits resulted from crosses without the mentor pollen, IvP101, while crosses using IvP101 yielded 1923 fruits. The difference in rates between the two groups is statistically significant (chi-square test p -value: $6.820\text{e-}91$), emphasizing the positive impact of the double pollination with IvP101 on fruit formation.

Out of these fruits, 3060 embryos were rescued and cultured under in vitro conditions (Table 1). Embryo formation showed variation based on cross type, classified by the wild species used and the employment of double pollination with IvP101. Cultured embryos comprised 25% globular, 37% heart-shaped, and 38% torpedo-shaped (data not shown). The success rate of developing viable plants from the rescued embryos differed based on the embryo stage, with heart- and torpedo-shaped embryos demonstrating higher efficiency compared to the globular stage.

Furthermore, the impact of double pollination with IvP101, as the mentor pollen, on embryo recovery was examined. The Kruskal–Wallis rank sum test revealed no significant difference in the number of rescued embryos among the different cross combinations with and without IvP101 ($H = 0.0109$, $df = 1$, $p = 0.9168$). However, it was observed that the percentage of ER hybrids surviving was lower in the crosses that used IvP101. For instance, the percentage of hybrids that

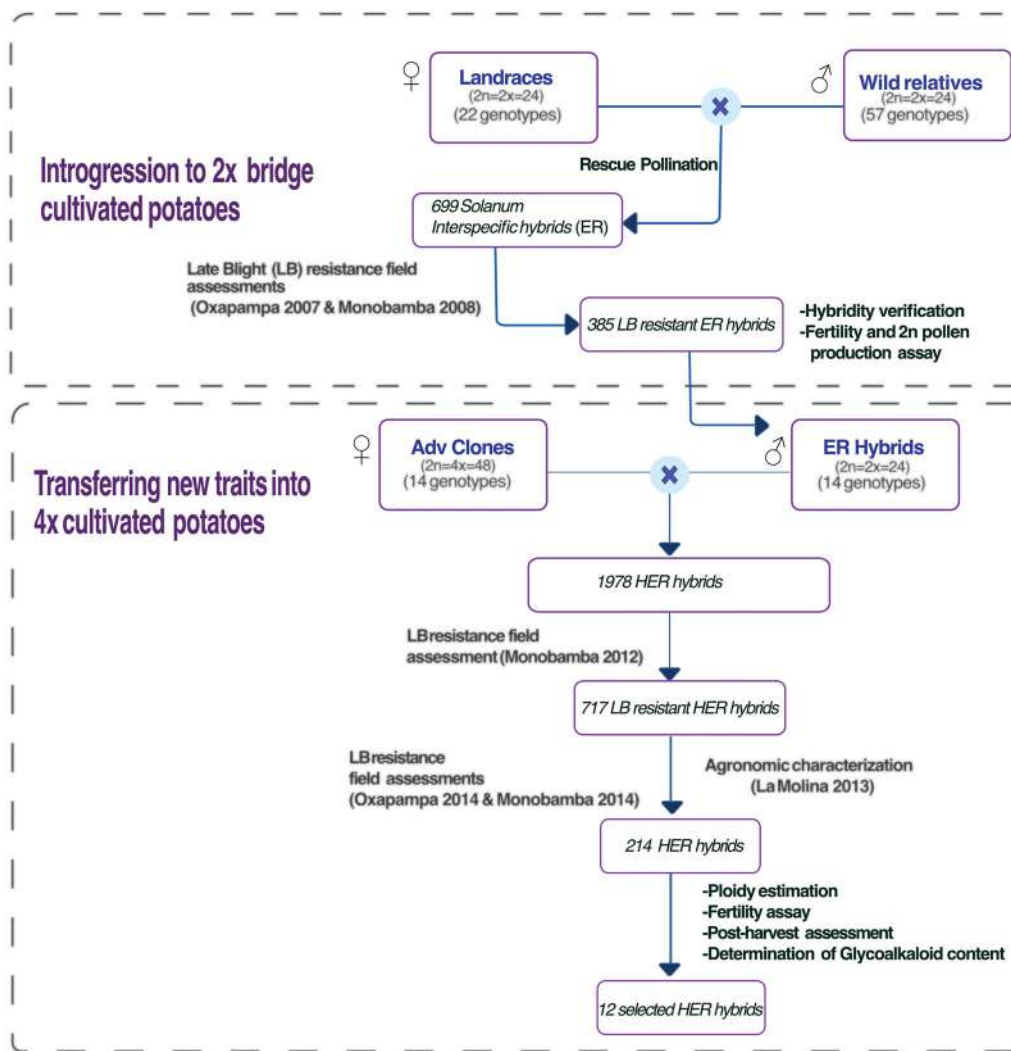


FIGURE 1 Outline of the pre-breeding workflow process used in this study, from wild relatives to the development of late blight-resistant pre-bred stocks. ER, embryo rescue; HER, hybrids from crosses with ER.

TABLE 1 Summary of the rescue pollination of hybrids between diploid landrace cultivars and wild tuber bearing relatives ($2x \times 2x$) from Piurana, Tuberosa, and Megistracroloba Series.

Cross combination ^a	No. of flowers pollinated	No. of fruits	No. of embryos rescued	Surviving ER plantlets (%)
Tbr clones \times <i>chq</i>	831	245	666	34
Tbr clones \times <i>chq</i> + IvP101	684	399	916	27
Tbr clones \times <i>pcs</i>	507	96	117	9
Tbr clones \times <i>pcs</i> + IvP101	385	235	97	3
Tbr clones \times <i>pur</i>	661	144	106	34
Tbr clones \times <i>pur</i> + IvP101	617	398	208	13
Tbr clones \times <i>cjm</i>	1422	370	500	23
Tbr clones \times <i>cjm</i> + IvP101	1388	884	442	5
Tbr clones \times <i>sgr</i>	12	2	8	100
Tbr clones \times <i>sgr</i> + IvP101	7	7	0	0
Total	6514	2780	3060	23

Abbreviation: ER, embryo rescue.

^aTbr refers to the group of pistillate parents, which includes diploid landrace cultivars and bred stocks.

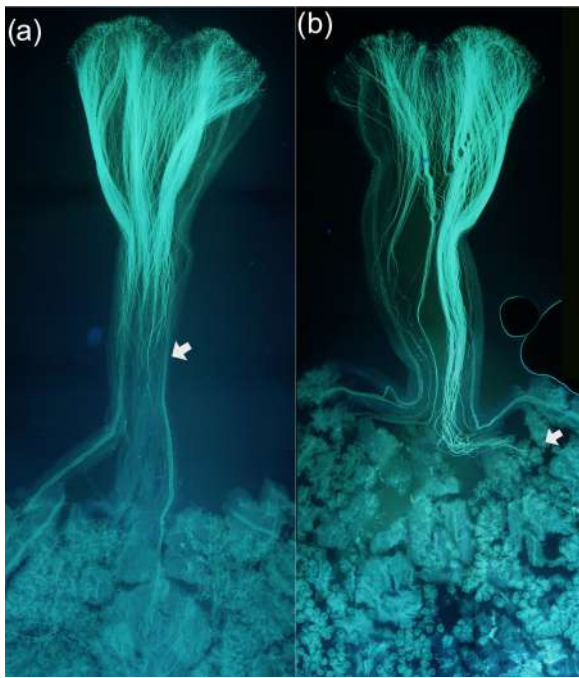


FIGURE 2 Examples of pollen–pistil relationships in crosses between diploid cultivated lines and wild relatives from Series Piurana. (a) Arrested pollen tubes at the stigma (CIP703280 × 762126.217), and (b) pollen tubes reaching ovules (CIP703280 × 762126.217 + IvP101). The white arrows indicate the site of arrest of the pollen tubes.

survived from the crosses with and without IvP101 was 27% and 34% for *chq*, 5% and 23% for *cjm*; 3% and 9% for *pcs*; 13% and 34% for *pur*; and 0% and 100% for *sgr* (Table 1).

The majority of in vitro plantlets derived from the cultured embryos exhibited normal development of roots and shoots. However, some seedlings showed good development of the root system but failed to develop apical shoots (Figure 3d). Despite being able to grow in the culture medium, the abnormal embryos exhibited slower growth compared to the average embryos (Figure 3e). These embryos were ultimately unable to develop into viable plants and eventually died. The rate of surviving ER plantlets accounted for 23% (i.e., 699 ER plantlets) of the total embryos rescued (Table 1).

3.3 | Ploidy, cytological characterization, and hybridity assessment of ER hybrids

All 699 ER hybrids were classified as diploids by chloroplast counting (data not shown). Diploidy was further confirmed in a subset of 165 ER hybrids via flow cytometry by Quispe (2011). The wild parents exhibited high pollen viability, with an average viability of 80%. Out of the 699 ER hybrids, only 148 produced enough pollen for the viability assay. Fifteen of these ER hybrids showed unviable pollen or sterile pollen. Additionally, 32 presented flower bud abortion. The pollen viability of the ER hybrids that produced and retained flowers

ranged from 40% to 94% (average = 73.3%). The frequency of unreduced pollen among the ER hybrids varied from 0% to 4%. Quispe (2011) characterized 165 hybrids out of the 699 ER hybrids using two polymorphic SSR markers, confirming 124 as hybrids from the corresponding cross combinations.

3.4 | Late blight resistance of ER hybrids

To select the most promising genotypes for subsequent crossing, ER hybrids and their wild parents were evaluated for LB resistance under endemic field conditions in Oxapampa and Monobamba in 2007 and 2008, respectively (Figure 4). This assessment involved 546 ER hybrids that exhibited good growth development and 56 wild species parents. To ensure a representative sample, 385 ER genotypes with moderate to high LB resistant levels in both locations were selected. The controls maintained their known level of LB resistance in both locations.

Out of the 385 selected ER genotypes, the most promising candidates were identified as 304 hybrids from *chq*, 47 from *cjm*, 25 from *pur*, eight from *pcs*, and one from *sgr*. Among them, 14 LB-resistant ER hybrids were chosen as the best candidates for use in the interploidy (4x-2x) crosses as staminate parents based on their high unreduced pollen frequency (Table S1). The hybridity of the 14 ER hybrids was confirmed through cross-checking with the hybridity results reported by Quispe (2011). Of these 14 ER hybrids, 10 were hybrids from *chq*, two from *pcs*, one from *sgr*, and one from *cjm*.

3.5 | Success rate in unilateral sexual polyploidization

Out of the 196 possible interploidy cross combinations (14 4x breeding lines and varieties, referred to as Adv Clones, crossed with 14 ER hybrids), a total of 64 successful cross combinations—referred to as families, were obtained. In some cases, few combinations were generated due to asynchrony in flowering, this was the case in crosses using the *Hyb sgr*. The fruit-setting rate in the interploidy crosses varied depending on the cross combination, ranging from 13.7% to 61.1%. The fruit-setting rate was higher in crosses between Adv Clones and *Hyb pcs* (61.1). But this was due to the few pollinations performed using this hybrid (Table 2). Each cross combination has a range of 1 and 151 seeds each. In total, there were 1978 seeds.

3.6 | Late blight resistance of HER hybrids

The first field assessment for LB resistance in Monobamba 2012 revealed significant differences among the HER hybrids (Figure 5). While most of the ER parents of the HER

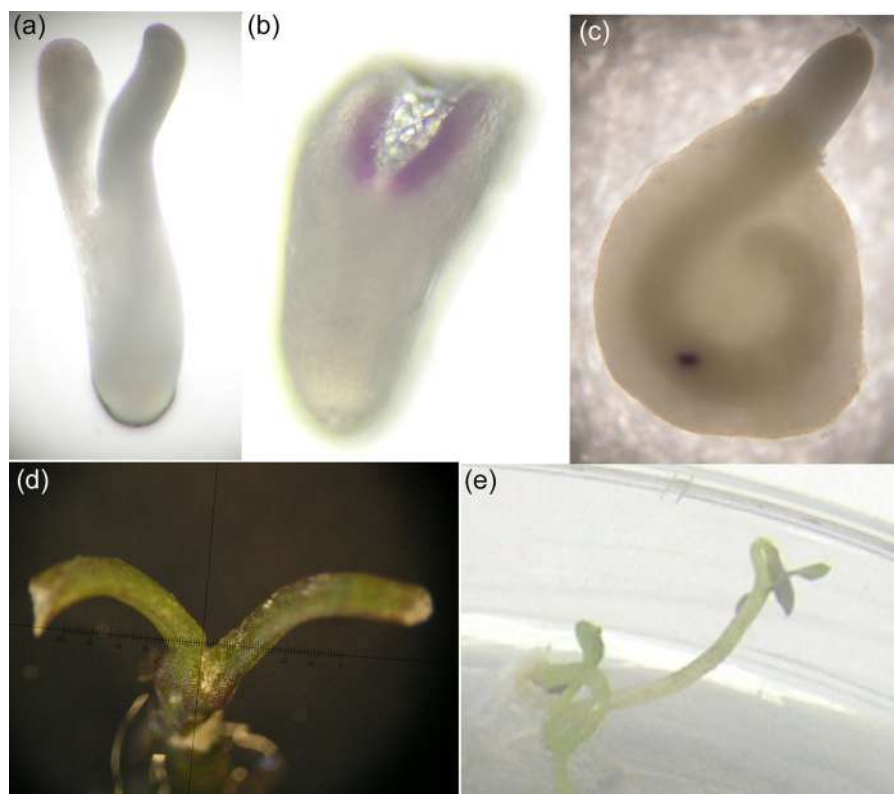


FIGURE 3 (a) Torpedo-shaped embryo, (b) torpedo-shaped embryo with a purple spot marker, (c) embryo-spot marker derived from IvP101 (HI) is visible at seed stage, (d) plantlet without shoot apical meristem, and (e) plantlet after 1 month of in vitro culture. HI, haploid inducer.

TABLE 2 Crossability behavior in the interploidy crosses (4x-2x).

Cross combination ^a	No. of pollinated flowers	Successful cross combinations		Seed/fruits ^c	Range of individuals generated per combination
		No.	Mean (%) ^b		
Adv Clones (12) × Hyb <i>chq</i> (10)	764	35	23.9 ± 0.5	4.9 ± 3.9	1–136
Adv Clones (9) × Hyb <i>pcs</i> (2)	190	11	13.7 ± 0.2	7.4 ± 1.4	1–48
Adv Clones (14) × Hyb <i>cjm</i> (1)	354	14	59.9 ± 0.3	4.1 ± 13.5	5–151
Adv Clones (4) × Hyb <i>sgr</i> (1)	36	4	61.1 ± 0.1	0.6 ± 1.9	1–5

Abbreviation: Adv Clones, advanced 4x lines.

^aCross combinations are indicated as pistillate × staminate. The number of genotypes involved in the cross given in parenthesis.

^bMean of the berry-setting rates (percent fruit/flowers) over all cross combinations with standard deviation.

^cMean among successful cross combinations with standard deviation.

maintained their expected resistance, ranging from resistant to HR, ER parent *cjm* (CIP506108.2) exhibited an MR level, which was lower than the resistance observed in previous assessments. This discrepancy may be attributed to the limited number of replications ($n = 2$) in this specific field trial. In contrast, the 4x parents displayed diverse levels of LB resistance.

In Figure 5, the distribution of BLUPs-predicted rAUDPC values indicates the segregation of two types of resistance in the HER progenies: complete resistance and quantitative resistance. If the resistance had been caused by a dominant *Rpi* gene in the ER parent, it would have been expected that

the entire 4x progeny would have inherited the resistance phenotype. Therefore, since the resistance phenotype ranges from complete resistance to susceptibility, it is possible that the resistance provided by the ER parent is quantitative. Additionally, it is possible that the tetraploid pistillate parents may have contributed to the resistance in the HER hybrids.

Out of the total of 717 promising HER hybrids, ranging from resistant to HR, 355 were selected for further assessments, including evaluations of fertility, ploidy, agronomic characteristics, and LB resistance.

Consequently, additional assessments of field resistance were conducted to confirm their resistance. Specifically,

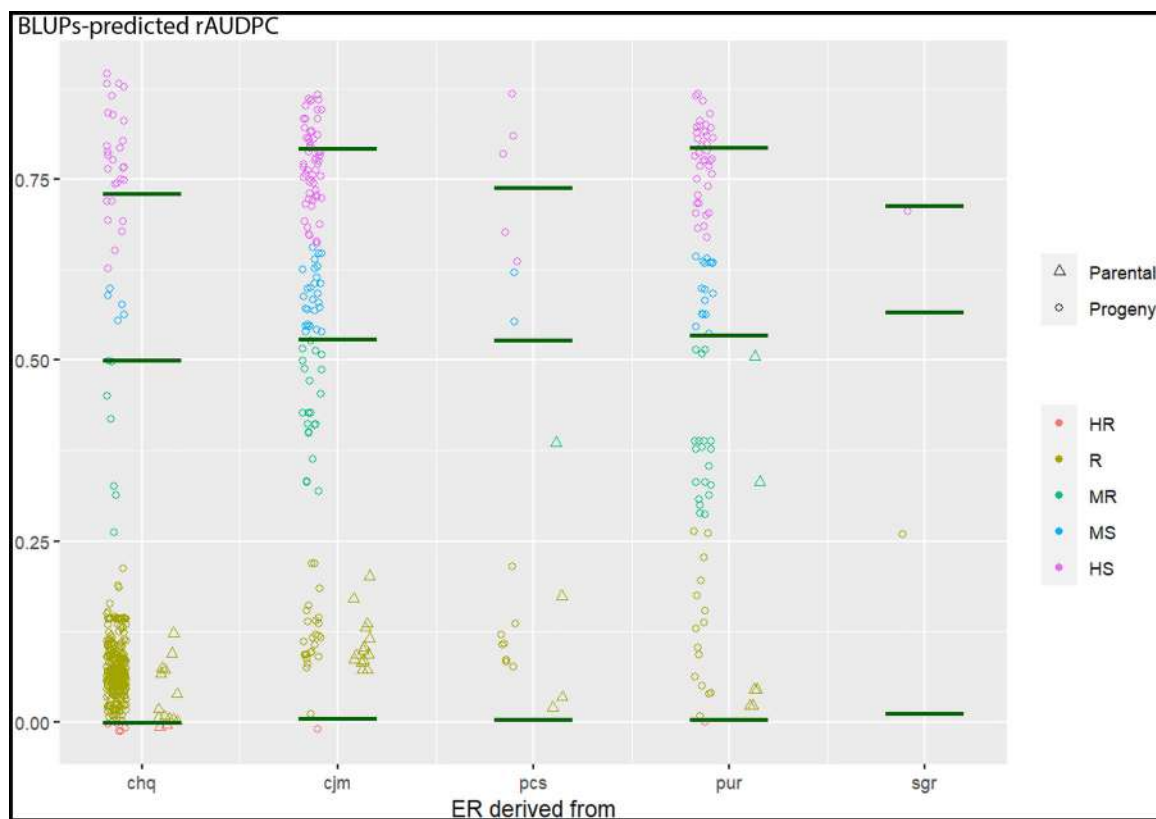


FIGURE 4 BLUPs-predicted rAUDPC distribution of ER hybrids and their wild parents grouped based on late blight resistance in two locations, Oxapampa and Monobamba, 2007 and 2008 respectively. Solid lines correspond to controls based on their resistance. The *x*-axis represents the contribution of the wild parent. Among the total number of ER hybrids tested, seven individuals were highly resistant (HR), 337 were resistant (R), 41 were moderately resistant (MR), 41 were moderately susceptible (MS), and 120 were highly susceptible (HS). BLUPs, best linear unbiased predictions; ER, embryo rescuer; AUDPC, relative area under disease progress curve.

during the rainy season in 2014, 270 HER hybrids underwent a second season of field exposure in Monobamba, while 154 underwent the same in Oxapampa. The resistance trend observed in these trials was similar to the results obtained in the initial assessment (Figure 6). In both 2014 LB resistance evaluations, the ER parent *cjm* (CIP506108.2) displayed a higher level of resistance than in 2012. This result supports the resistance assigned for this ER hybrid in the previous assessments. As expected, the mean rAUDPC of the 2x wild parents was lower than of the 4x parents. Two hundred fourteen HER hybrids with moderate to highly LB resistant levels were selected for further evaluations. Of these 214, 113 were HER hybrids derived from *chq*, 69 from *cjm*, 25 from *pcs*, and seven from *sgr*.

3.7 | Ploidy, cytological, agronomic, and quality characteristics of HER hybrids

Ploidy estimation of the selected LB resistant HER hybrids by chloroplast counting revealed that all cross combinations except Adv Clones × *Hyb sgr* yielded progeny comprising 3x

and 4x individuals. Adv Clones × *Hyb sgr*, on the other hand, only produced 4x individuals. Out of the 355 HER hybrids examined, 326 were tetraploids, while 29 were triploids.

Among the HER hybrids, a range of flower development and retention patterns was observed, with 46 plants displaying bud abortion or floral abscission. Of the 311 clones blossoming (data not shown), 168 showed moderate to high pollen viability based on the scale described by Ordonez et al. (2017).

Agronomic evaluation performed on selected HER hybrids, which were moderate to HR, revealed that those derived from *cjm* significantly out-yielded their tetraploid parents. For instance, the average yield for these HER hybrids was 0.9 kg per plant, compared to 0.8 kg per plant for the 4x parents (Table 3). The 4x parents of the HER hybrids are elite clones from the CIP potato breeding program. These elite clones are adapted to the highland tropics environment and have the maturity around 90–100 days. In the target environments they are as early as the local regular cultivars. HER hybrids derived from *chq*, *pcs*, and *sgr* followed closely with an average yield of 0.7 kg per plant. Furthermore, the variable number and length of stolons documented in the HER hybrids could be attributed to the contribution of their wild

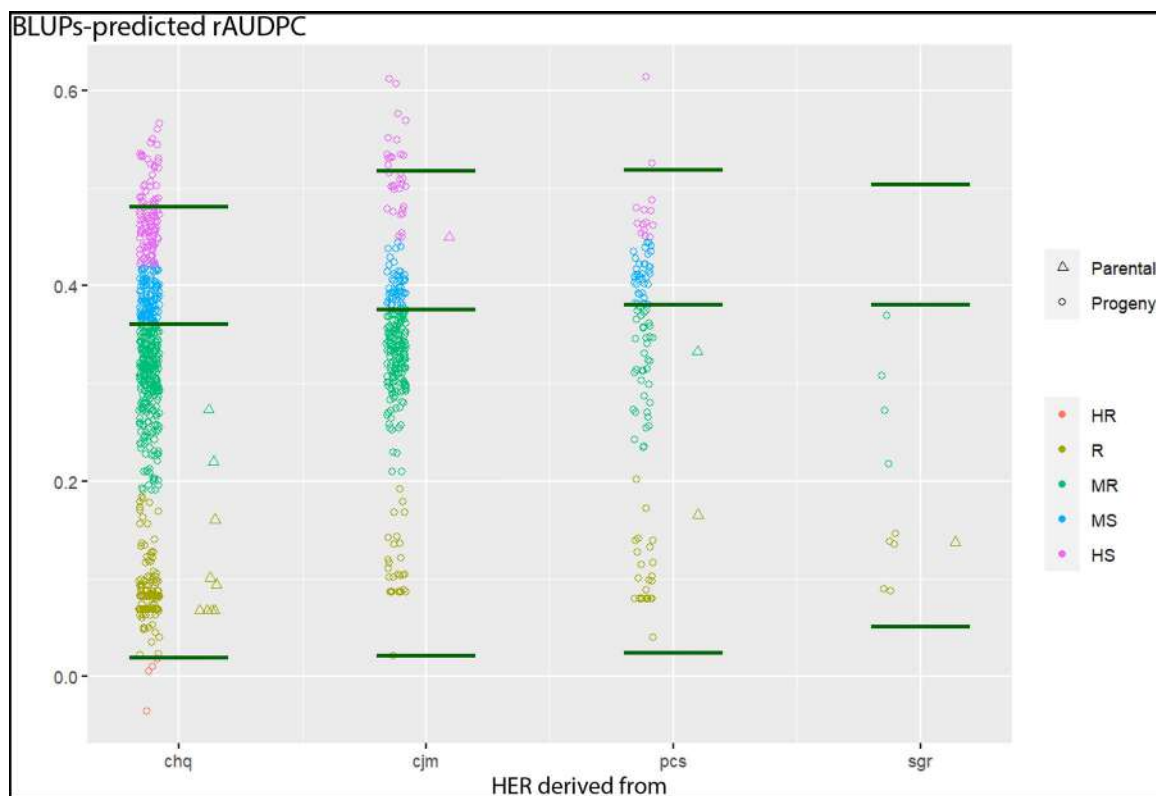


FIGURE 5 BLUPs-predicted rAUDPC distribution of HER hybrids and their wild parents grouped based on late blight resistance in Monobamba (2012). Solid lines correspond to controls based on their resistance. The x-axis represents the contribution of the wild parent. Among the total number of HER hybrid tested, four individuals were highly resistant (HR), 215 were resistant (R), 498 moderately resistant (MR), 206 were moderately susceptible (MS), and 156 were highly susceptible (HS). BLUPs, best linear unbiased predictions; HER, hybrids from crosses with ER; rAUDPC, relative area under disease progress curve.

TABLE 3 Mean values of yield and number and length of stolons of the HER hybrids and their 4x parents.

Parents/cross combination	N ^o hybrids tested	Yield (kg/plant)	Number of stolons ^a	Length of stolons (cm) ^a
Adv Clones (4x Parents)	14	0.8 ± 0.3b	1.3 ± 0.6c	1.5 ± 0.8c
Adv Clones × Hyb <i>chq</i>	232	0.7 ± 0.3b	4.0 ± 1.9a	4.0 ± 1.8a
Adv Clones × Hyb <i>pcs</i>	55	0.7 ± 0.3b	3.4 ± 1.5a	3.6 ± 1.4a
Adv Clones × Hyb <i>cjm</i>	60	0.9 ± 0.3a	2.5 ± 0.9b	2.0 ± 1.2b
Adv Clones × Hyb <i>sgr</i>	8	0.7 ± 0.2b	2.1 ± 1.4b	2.0 ± 1.4b

Abbreviation: HER, hybrids from crosses with embryo rescue.

^aValues within a column with differing lowercase letters are significantly different at $p \leq 0.05$.

ancestors. Specifically, HER hybrids derived from *chq* and *pcs* had a higher mean value of both, number and length of stolons (average = 4.0 and 4.0; 3.4 and 3.6, respectively) compared to those derived from *cjm* and *sgr* (average = 2.5 and 2.0; 2.1 and 2.0, respectively).

Twenty-eight HER hybrids were selected based on a combination of their levels of LB resistance, yield, and pollen viability. Selected genotypes were further assessed for dry matter and glycoalkaloid contents. Out of these, 17 showed dry matter contents ranging from 18% to 27%, and glycoalkaloid contents less than 19 mg 100 g⁻¹ fresh

weight (FW), with hybrids from *cjm* showing the lowest glycoalkaloid contents (Table 4). Remarkably, among these, there were 12 hybrids with levels below 6–7 mg 100 g⁻¹ FW that can be suggested for breeding purposes (Table S3). High glycoalkaloid content is a characteristic of many wild potato species, and varieties with >20 mg 100 g⁻¹ FW in tubers are considered unacceptable for human consumption (Friedman et al., 1997). Additionally, within the tubers, samples with the peel showed a higher concentration of glycoalkaloids compared to samples without the peel (Table 4).

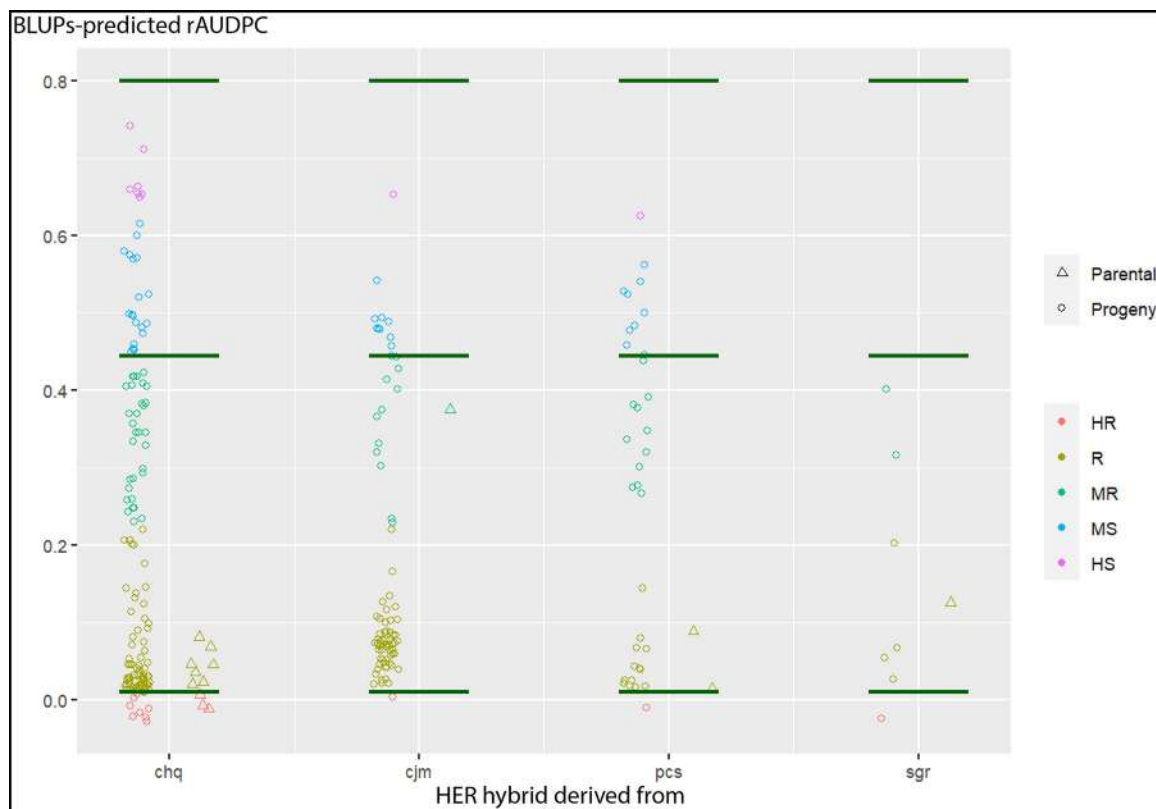


FIGURE 6 BLUPs-predicted rAUDPC distribution of HER hybrids and their wild parents grouped based on late blight resistance in Monobamba and Oxapampa in 2014. Solid lines correspond to controls based on their resistance. The x-axis represents the contribution of the wild parent. Among the total number of HER hybrid tested, 12 individuals were highly resistant (HR), 147 were resistant (R), 55 were moderately resistant (MR), 40 were moderately susceptible (MS), and nine were highly susceptible (HS). BLUPs, best linear unbiased predictions; HER, hybrids from crosses with ER; rAUDPC, relative area under disease progress curve.

TABLE 4 Glycoalkaloid levels and dry matter percentage in a subset of 17 HER hybrids grown under field conditions in La Molina in 2013.

Cross combination	No. of hybrids tested	Dry matter range (%)	Total glycoalkaloids in tubers (mg 100 g ⁻¹ fresh weight)	
			With peel	Without peel
AdvClones × Hyb <i>chq</i>	4	18.4 – 24.0	5.2 – 18.8	2.3 – 7.4
AdvClones × Hyb <i>cjm</i>	9	21.6 – 27.1	2.3 – 12.1	1.4 – 8.9
AdvClones × Hyb <i>pcs</i>	4	21.2 – 24.7	18.8 – 51.2	4.1 – 35.1

Abbreviation: Adv Clones, advanced 4x lines.

4 | DISCUSSION

In this study, we described the roadmap followed by the CIP prebreeding program to generate new and valuable potato stocks by incorporating LB resistance from underutilized PWR into advanced tetraploid backgrounds. Based on ploidy, fertility, LB resistance, desirable tuber characteristics, and low glycoalkaloid content, these well-documented genotypes can be recommended as pre-bred stocks to continue the potato breeding process. In fact, eight of the most promising HER selections were evaluated with the participation of

Andean farmers to provide breeders with additional information on characteristics required for adoption and consumption. As a result, two HER selections have been suggested for direct use as improved potato varieties under tropical highland conditions of Peru (Scurrah et al., 2019). Notably, one of these selections has recently been released as a LB-resistance variety named “CIP-Matilde” (CIP, in preparation).

Rescue pollination has been previously employed to transfer favorable alleles from PWR that cannot be directly crossed with cultivated potatoes. HIs, such as *phu1.22*, *IvP35*, or *IvP101*, are commonly used as mentor pollens due to their

homozygous purple marker (Hermsen & Verdenius, 1973), which makes it easier to sort out embryos without or with the genetic contribution of the mentor pollinator. Rescue pollination is particularly important when the aim is to circumvent restricting barriers, such as the EBN. While previous studies (Ramon & Hanneman, 2002; Singsit & Hanneman, 1991; Watanabe et al., 1995) highlighted mentor pollen's role in preventing premature fruit drop and promoting hybrid seed formation, the impact of double pollination on embryo survival and fruit formation remains debated (Yermishin et al., 2014). In our study, double pollination with IvP101 increased fruit formation, but had no effect on the number of rescued embryos or surviving plantlets. Despite this, we obtained 699 rare sexual hybrid combinations through rescue pollination. Additionally, our findings support the relationship between embryo stage and successful development, as shown by Singsit and Hanneman (1990). Advanced embryos (heart- and torpedo-shaped) have higher rescue success and plant formation rates compared to globular embryos (Lester & Kang, 1998).

The identification and characterization of ER sexual hybrids began at the embryo stage by only culturing embryos without the HI spot marker. During the culture, we documented the presence of hybrids between 2x and the HI IvP101, these displaying embryo spots (Figure 3c). We did not identify either parthenogenotes or tetraploid plants, as cautioned by Watanabe et al. (1995). We did observe plantlets lacking the apical meristem, however, their embryos had normal morphology. At the plant stage, examination of pollen revealed that blooming ER hybrids have moderate fertility, with some displaying low pollen shed. However, a significant number of them failed to flower. Camadro et al. (2004) reported that male sterility in F1 hybrids is a critical post-zygotic barrier that hinders further hybridization processes. This sterility could be caused by deleterious interactions, or abnormal meiotic products from new chromosomal rearrangements (Jackson & Hanneman, 1999; Yoon et al., 2006). The ER hybrids reported here are unique, not only because of their recovery rate, but also some of them also inherited desirable traits, such as the tuber appeal (e.g., high dry matter) of the cultivated 2x potatoes and the LB resistance from the wild species. These traits, along with their unreduced pollen production make them promising materials for use in a diploid inbred-based hybrid breeding strategy aimed for pyramiding LB resistance genes.

We observed significant variation in berry set in the interploidy (4x-2x) crosses. Among the five wild relatives studied, ER hybrids derived from *chq* had the highest success rate in crosses with 4x lines but resulted in very low seed set per fruit. Our results suggest that species-specific compatibility may play a critical role in limiting the transfer of novel resistant alleles from the ER selection stocks. While this species-specific compatibility hampered the gen-

eration of more hybrids derived from *pcs*, the opposite occurred with *chq*, resulting in more hybrids being obtained from this species, even though both belong to the same series, Piurana.

Unlike the ER progeny, which all turned out to be diploids, the HER hybrids encompassed a substantial number of tetraploid hybrids in addition to triploid hybrids. Even though this reflected the ploidy of only a subset of 355 LB resistant HER hybrids selected after the first field assessment. Our findings differ from Graebner et al. (2022), who reported a higher frequency of triploid clones than tetraploid ones in interploidy (4x-2x) crosses. This confirms that the ER hybrids have a higher frequency of functioning unreduced pollen. As a result, we were able to select several high-yielding 4x hybrid genotypes within hybrid families from a wide range of interploidy crosses. Several studies have pointed out that clones arising from interploidy (4x-2x) crosses exhibit a heterotic advantage, often exceeding the yield of 4x cultivars. The positive effect of sexual tetraploidization on yield seems to be accompanied by a decrease in fertility, but this effect is linked to the maternal cytoplasm type (Hanneman & Pelouquin, 1981). For instance, Buso et al. (1999) observed strongly heterotic responses in a tetraploid progeny derived from interploidy (4x-2x) crosses, which were attributed to the first meiotic division restitution. Moreover, sexual polyploidization through unreduced gametes is known to maintain and transmit parental heterozygosity and epistasis, which is beneficial in maximizing heterosis for yield (De Jong et al., 1981; Gopal, 2014).

The introduction of novel *Rpi* genes through conventional or genetic engineering-based breeding methods is essential for improving and maintaining durable resistance to potato LB in new cultivars. The diversity of resistance genes of PWRs, such as those endemics to LB-prone areas, highlight the importance of mining novel genes through their use. For instance, Rogozina et al. (2021) demonstrated that hybrids combining germplasm of more than three PWR were HR to *P. infestans*, suggesting that stacking *Rpi* genes can help achieve nonspecific LB resistance in potato cultivars. In a different approach, Keijzer et al. (2022) made some PWR (e.g., *Solanum circaeifolium* var. *capsibaccatum*, *S. chacoense* and *S. venturii*) available for breeding of LB resistant varieties by repeated backcrosses to 4x varieties and further selections within the Bioimpuls project scope. The 11-year-old project reported by Keijzer et al. (2022) represents a significant effort to develop LB-resistant potato varieties through conventional breeding methods, with the use of PWR playing a crucial role in achieving their goal. Similarly, in our two-stage study, which was conducted at the diploid and tetraploid levels for ER and HER hybrids, respectively, we identified hybrids with high levels of LB resistance. However, additional research is required to confirm the novelty of their resistance. Our findings indicate that

the LB resistance of the HER hybrids was also contributed by the 4x elite parents and was phenotypically indistinguishable from the resistance originating from the wild source. Further research is needed to identify the genomic region(s) responsible for the resistance in the HER hybrids. The R gene enrichment and sequencing (Jupe et al., 2013) and differential inoculation with *P. infestans* testers would be valuable to confirm their novelty with respect to resistance sources commonly available or already present in advanced populations. For instance, the resistance in one of the sources, *S. paucissectum*, has been mapped to chromosome 11 in potato (Villamon et al., 2005).

Though encouraging, the results of using unadapted germplasm also led to carry over of undesirable traits of the PWR, such as poor tuber appeal, numerous long stolons, or high glycoalkaloid contents of some hybrids, which remain to be addressed in further intercrossing or backcrossing to additional agronomically adapted lines and varieties. Nonetheless, as pointed out by Jansky et al. (2021) breeding approaches that apply excessively strong selection pressure against undesirable agronomic traits can eliminate wild germplasm in early stages of selection. This was also stressed by Andino et al. (2022) in their study of introgressing bacterial wilt resistance from the PWR, *Solanum commersonii*. Therefore, we have maintained several progenies of each HER hybrid type and recommend intercrossing along with molecular tagging to maximize the likelihood of broadening the base of the resistance in future breeding with them. Our results argued with traditional concepts that wild species are best for incorporating specific traits such as disease resistance, for which they have been screened, as they can also be considered as contributors to yield and tuber quality traits. The value of exotic germplasm for improving traits not evident from their phenotype and strategies for their use were highlighted by Tanksley and McCouch (1997).

Two major achievements emerged from this research: the establishment and characterization of promising sexual (ER) hybrids from underutilized and endemic PWRs, and the selection of promising 4x hybrids (HER) hybrids combining useful agronomic and resistance traits. Although genetic data are limited and we cannot demonstrate the presence of wild relatives' genomes in the HER hybrids, we have confidence in their containing novel diversity and likely novel sources of LB resistance due to our low selection pressure and the persistence of some wild species traits, such as long stolons. The selected HER hybrids and their pedigree parents are currently undergoing multiplication to enable thorough characterization of their LB resistance type and spectrum. The ER and HER hybrids are crossing-friendly stocks that can be used in breeding strategies and the new diploid inbred-based hybrid breeding approach, thus reducing the time for developing new varieties and maximizing the use of genetic variability available in the PWRs.

5 | CONCLUDING REMARKS

Our interdisciplinary approach to establishing and characterizing sexual hybrids is the first step toward efficiently transferring LB resistance from a new group of underutilized wild distant species to the cultivated potato. We developed new 4x sources of LB resistance by first introgressing resistance from distant wild relatives into 2x cultivated *S. tuberosum* through rescue pollination, and then transferring the resistance to adv clones via polyploidization using unreduced gametes. These LB-resistant tetraploid hybrids exhibit promising agronomic and quality attributes, with the hybrids from *S. cajamarquense* particularly standing out among the sets of HER genotypes generated.

AUTHOR CONTRIBUTIONS

Benny Ordoñez: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing—original draft, Writing—review & editing, **Mariela Aponte:** Data curation, Formal analysis, Validation, **Hannele Lindqvist-Kreuze:** Validation, Writing—original draft, Writing—review & editing, **Merideth Bonierbale:** Conceptualization, Funding acquisition, Supervision, Writing—original draft, Writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Of the 12 HER hybrids identified in this study, six are currently available for international distribution. The distribution is subject to the signing of the Standard Material Transfer Agreement (SMTA) and any additional clauses that may

apply. Interested parties can request these hybrids from CIP Lima, Peru, by emailing CIP-Germplasm@cgiar.org. The remaining six HER hybrids, as well as the ER hybrids and wild accessions, are still undergoing further evaluation and will be made available for distribution at a later date.

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