Strategic Breeding Approaches: Harnessing Molecular and Phenotypic Markers to Overcome Quinoa's Flower Morphology Bottlenecks

Prashant Vikram

pvikramseedwheat@gmail.com

International Center for Biosaline Agriculture

HIFZUR RAHMAN

International Center for Biosaline Agriculture

Lovely Mehta

International Center for Biosaline Agriculture

Sakshi Vidit Jain

International Center for Biosaline Agriculture

Sugandha Asthana

International Center for Biosaline Agriculture

Sowkat Nabi Rather

International Center for Biosaline Agriculture

Murali Gumma Krishna

International Crops Research Institute for the Semi-Arid Tropics

Srinivasan Samineni

International Center for Biosaline Agriculture

Gyan Prakash Mishra

Indian Agricultural Research Institute

Rakesh Kumar Singh

International Center for Biosaline Agriculture

Article

Keywords: Quinoa, Pseudocereals, breeding, marker-assisted breeding, genetic enhancement

Posted Date: April 27th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9391447/v1

Additional Declarations: No competing interests reported.

Abstract

Quinoa represents a valuable gift from the "New World" to the "Old World." Although some breeding programs exist worldwide, they have seen limited growth and impact on its genetic improvement. Large-scale hybridization, hindered by the crop's complex floral morphology, remains the primary bottleneck in quinoa breeding. This study compared manual and natural crossing schemes, incorporating hybridity testing with morphological and molecular markers. The results indicate that a facilitated open-pollinated strategy combined with hand emasculation and hybridity confirmation using morphological or indel markers offers a feasible approach to establishing a quinoa breeding pipeline. We also concluded that characterising panicles by the arrangement of hermaphrodite and pistillate flowers could be a game-changer for improving breeding efficiency. Additionally, interspecific crosses (Chenopodium quinoa × Chenopodium giganteum) achieved efficiencies of 5–26%. Both inter- and intraspecific crosses can feasibly generate variation for genetic improvement in quinoa. Breeding quinoa for Asian environments should follow a step-wise strategy: (1) characterize flowering in crossing blocks, (2) apply natural crossing or hand emasculation, (3) confirm hybridity using morphological or molecular markers, (4) pursue genetic enhancement, and (5) select for traits of interest.

1. Introduction

Quinoa (*Chenopodium quinoa*) is one of the oldest crops of the American continent, believed to have originated and cultivated in the Andean region, mainly in Peru, Bolivia, and Chile. Quinoa can be considered a new gift of the ‘new world’ to the ‘old world’, like the potato and tomato. Quinoa has earned special attention worldwide due to its exceptional nutritional quality, health benefits and ability to adapt to marginal environments (Jacobsen, 1997, 2003, 2015).

Quinoa, a pseudocereal due to its grain structure, is an annual dicotyledonous species that belongs to the family *Amaranthaceae* (formerly *Chenopodiaceae*). Quinoa is exemplary for amino acid composition and offers a remarkable balance of oil (4–9%), protein (averaging 16%, with high nutritional value), and carbohydrates (64%) (James 2009). It can be used to make flour in the same way as cereals due to its high starch content (51–61%). Quinoa grain is free of gluten, which has facilitated the creation of various products for people with celiac disease (i.e., those who are allergic to gluten) (Singh 2019, Amin et al. 2022).

Quinoa is one of the oldest crops but its limited spread happened in the past few decades (Bazile et al. 2016). The major reason for the limited spread is primitive breeding efforts, mostly reported from Bolivia and Peru (Zurita-Silva et al. 2014). Although some recent breeding studies have reported the development of high-yielding varieties adapted to temperate regions and high latitudes of Europe, North America, and China, most of these were limited to mutation breeding or mass selection (Murphy et al. 2016, Patirange et al. 2022). Many Asian and African regions are continuously making efforts to increase the genetic biodiversity of the quinoa cropping system by hybridization to develop high-yielding, sweet and improved varieties, with limited success (Murphy et al. 2016).

The biggest challenge in large-scale quinoa breeding is due to its very small florets and the presence of both hermaphrodite and female flowers (out-crossing 0.5% to 17%) on the same panicle (Murphy et al. 2016). Due to the complex arrangement of the hermaphrodite and pistillate (female) flowers, small size of flowers and fast progression of flowering within infloresence it becomes very difficult to perform hand emasculation efficiently (Paterson et al. 2015). Hot water treatment is another method followed for emasculation in which hot water (~ 45°C) application is done on the inflorescence in a way to inactivate the pollens while keeping ovaries and stigma safe, however, this method is also reported to damage the inflorescence (Fleming et al. 1995, Sha 2013). Though it is time-consuming, imparting male sterility can be an alternative method of creating hybrids. Some male sterility sources have been identified in quinoa including cytoplasmic male sterility (CMS) germplasms along with their restorer genes (Simmonds 1971, Ward and Johnson 1994). It is important to note that heterosis has not been reported in quinoa so far, so, application of CMS system might not be very feasible option, hence not pursued further at commercial scale.

Quinoa is also known for its highly variable outcrossing rates across different accessions (Murphy et al. 2016) that can be utilized for enhancing the quinoa breeding program efficiency. Identification of the true F_1s is another important concern in quinoa breeding. Broadly, there are two strategies implied for the identification of F_1 plants in quinoa i.e. using morphological and/or molecular markers. Traits such as axillary pigmentation, plant colour, seed colour and inflorescence can be used as diagnostic markers provided the male parent (pollinator) is homozygous for the dominant alleles (Paterson et al. 2015). On the other hand, molecular marker use in hybridity testing requires polymorphic markers between the two parents of the F_1 plants.

In the current study, efforts have been made to employ different crossing strategies to establish a robust quinoa breeding platform specifically for the newer environments including Asia/ Middle East.

2. Material and Methods

2.1. The quinoa crossing block

Quinoa breeding work was initiated using gene bank accessions stored in the International Centre for Biosaline Agriculture (ICBA), Dubai, UAE, in December 2020. A subset from these accessions was subjected to saponin estimation analysis and reported recently (Tabatabei et al. 2022). Low saponin accessions were shortlisted from the report of Tabatabaei et al. 2021, and, validated through reanalysis following afrosimetric method (Koziol 1991). Seven parents were found to be suitable donors for low saponin parents (Ames 19046, D12401, D12406, D 11912, D12377, Chen281 and Chen254). Based on visual observations, six other donors (Co 407, D12047, ICBA-Q5, BO 51, Titicaca and ICBA-Q3) were selected for grain yield and adaptive characteristics (Supplementary Figs. 1a-b). ICBA-Q5 and ICBA-Q3 are two lines selected at ICBA and were nominated as varieties in different countries (https://www.biosaline.org/news/2020-03-14-7046). Titicaca is a widespread variety of Europe; Co 407 is a variety from Colorado State University, USA, developed by mutation. D12047 and BO 51 were

identified as high-yielding accessions during field experiments for the GWAS study at ICBA fields in the 2020-21 trial (Rahman et al. 2024).

2.2. DNA extraction and PCR amplification

Genetic analyses were performed for the characterization of crossing blocks and testing of hybridity. DNA of the crossing block lines and F_1 plants were extracted following a modified CTAB method explained by (Cota-Sanchez et al. 2006). The InDel markers reported by (Zhang et al. 2017) were synthesized, and a parental polymorphism survey was carried out on the parental DNA. DNA was amplified using protocol explained by (Collar and Mackill, 2009). Reaction mixture of 20 µL included 10 µL of master (2X FIREPol® master mix), 0.5 µL of (0.1 µM) for each primer, and 1 µL of genomic DNA (50 ng/µL). The final volume was obtained using sterile distilled water. The PCR reaction was performed using a thermal profile with pre-denaturing for 5 min at 95°C, followed by 40 cycles at 94°C for 40 s, annealing for 40 s, and extension at 72°C for 30 sec, with a final extension time of 5 min at 72°C. PCR products were separated on a 3.0% agarose gel.

2.3. Genetic analysis

Data were scored as (1) for presence and (0) for absence for each sample under investigation. The polymorphism information content (PIC) was calculated according to the formula: PIC = 1 − Σpi^2 where pi is the frequency of the *i*th allele of the locus in eight genotypes (Anderson et al. 1993).The data was analysed with SIMQUAL program of NTSYS-pc (Version 2.02), and similarities between accessions were estimated using the Jaccard's coefficient calculated as J = A / (N - D), where A is the number of positive matches (that is, presence of band in both samples), D is the number of negative matches (that is, absence of band in both samples) and N is the total sample size including both the number of matches and unmatched. A dendrogram was created from the resultant similarity matrices using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method following the SAHN function of NTSYS-pc (Version 2.02).

2.4. Crossing and population advancement

Making crosses in quinoa is challenging because of its gyno-monoecious flowers and high resemblance of the hermaphrodite and female ones. Three different approaches were followed for making crosses by: (1) removing hermaphrodite flowers from the female parents, (2) forced synchronization of flowering and (3) natural crossing.

2.4.1. Crossing by removing hermaphrodite flowers

All 13 parents were sown in staggered planting mode in ICBA's green house during 5th December 2020 to 31st January 2021 at 15 days interval. At the peak flowering stage, hermaphrodite flowers were removed from the inflorescence leaving only female flowers on female parents and clubbed with that of male parent (in which hermaphrodite flowers were not removed) and covered with a glassine bag as illustrated in Supplementary Figs. 2 (a) and (b). Hermaphrodite flowers were observed and identified

using magnifying glasses (Supplementary Fig. 2c). One of the 13 parents was *Chenopodium giganteum* (Ames 19046) which was crossed with four *Chenopodium quinoa* accessions viz. D-12406, ICBA-Q3, Chen-254 and D-12377 (Supplementary Fig. 2b).

2.4.2. Forced synchronization of flowering

Five genotypes were identified for crossing through controlled flowering to force synchronization of anthesis: 1. ICBA-Q5, 2. D12401, 3. D12406, 4. Co 407 and 5. D12377. Selection of these five genotypes was done based on their unique characteristics including D12401: purple colour hermaphrodite flower, D12406: similar to D12401 but white colour hermaphrodite flowers, D12377: black seeded, Co 407: purple panicle and ICBA-Q5: Very early maturing. These five genotypes were sown in a clockwise fashion in the pot starting from #1 to #5 (Fig. 1).

They were first allowed to grow vegetatively, however, during flowering stage, early flowering panicles were cut with scissors in order to synchronize their flowering with other lines (Supplementary Fig. 2d). Quinoa plant has characteristic of continuously flowering for several weeks. This characteristic was exploited in this approach. We continued synchronization until when majority of the five plants flowered synchronously. After manual removal of panicles from the top, plants became usually short heighted and tender so that it became easy to tie (loosely) all of them and cover with a big glassine bag. Later on, each of the five lines were harvested from different pots and bulked. Seeds of individual five lines were sown in the next season in single plots so that crosses individuals can be visualized with the help of their parental distinctive diagnostic characteristics.

2.4.3. Natural crossing strategy through no emasculation method

In another approach, the pots with five genotypes (D12401, D12406, D12377, Co 407 & ICBA-Q5) with distinctive morphological characteristics as explained in the above section were kept just in front of the cooling pad of greenhouse so that slowly blowing wind from fan can facilitate movement of pollen from one genotype to another (Supplementary Fig. 2e). This way natural out crossing was facilitated. Each of the five genotypes were harvested from all pots and bulked for the next season sowing. As mentioned above, each line was harvested individually from different pots and formed five different bulks. Seeds of individual bulks were grown in the next season in individual plots to visualize probable crosses with the help of distinctive diagnostic characteristics. This method presents a slight modification in the ´no emasculation method´ as reported by (Emrani et al. 2020).

3. Results

3.1. Saponin estimation of the crossing block

Based on the saponin estimation analysis according to using afrosimetric method (Michael J Koziol 1991) parents for quinoa crossing block were declared. A total of seven genotypes were found suitable

to use as saponin free donor parents (Ames 19046, D12401, D12406, D 11912, D12377, Chen281 and Chen254), whereas six genotypes were used as parents for high grain yield and adaptive characteristics (Co 407, D12047, ICBA-Q5, BO 51, Titicaca and ICBA-Q3) based on in house experiments (ICBA Unpublished). Results are depicted in Supplementary Figs. 1(a) and (b). Unique characteristics of all the parents are delineated in the Table 1.

Table 1
Salient characteristics of the parents used for quinoa crossing block

Genotype Name	Key Trait	Remarks
Ames-19046	Saponin free-Super late	Preferred for Forage, different grain and plant type.
Chen-254	Saponin free	Medium
Chen-281	Saponin free	Medium
D-11912	Saponin free	Medium
D-12377	Saponin free	This is a heterogenous line producing two types of seed (brown & black), its f loral biology and flowering pattern need to be studied.
D-12401	Saponin free	Medium, this line has diagnostic hermaphrodite flower in certain environmental condition (pink color hermaphrodite flower, if grown in open light condition).
D-12406	Saponin free	Medium
BO-51	Yield & Adaptation Traits	Early
Co-407	Yield & Adaptation Traits	Early
D-12047	Yield & Adaptation Traits	This might be a segregating line, need not to use in breeding further.
ICBA-Q3	Yield & Adaptation Traits	Late
ICBA-Q5	Yield & Adaptation Traits	Early
Titicaca	Yield & Adaptation Traits	Very Early

3.2. Genetic profiling of parents in crossing block

Out of 36 indel primers studied 25 indels showed polymorphism among different accessions of crossing block. Figure 2 presents relationship among different crossing block accessions. Dendrogram clearly reveals that *Chenopodium giganteum* (Ames 19046) is distantly related with *Chenopodium quinoa* genotypes of crossing block. Broadly, there were two groups i.e. GP-I and GP-II; GP-I had three saponin free lines Chen254, D12406 and D 11912, whereas GP-II included ten diverse accessions including Ames 19046, D12401, D12377, Chen281, Co 407, D12047, ICBA-Q5, BO 51, Titicaca and ICBA-Q3.

3.3. Efficiency of three crossing methods

The hybridity testing of 15 cross combinations ascertained through seeds obtained from female flowers after removing hermaphrodite flowers from female parents, crossing efficiency was found in range of 5 to 71% (Table 2, Figs. 3a-c). In the interspecific crosses of '*Chenopodium quinoa × Chenopodium giganteum*' this Figure ranged 5–26%, whereas, among '*Chenopodium quinoa × Chenopodium quinoa*' crosses it was 10–71%. While making interspecific crosses *Chenopodium giganteum* was used as male parent because of its floral arrangement and multiple hanging branches. Among four interspecific crosses, 'ICBA-Q3 × Ames-19046' did not produced viable seed, whereas, other three successfully produced viable seeds. Maximum number of true hybrids were observed in 'D-12401 × BO-51' cross i.e. 15 out of 21 (71%), and minimum crossing efficiency was revealed in 'D-12401 × ICBA-Q3' as presented in Table 2.

Table 2

Table presenting the bi-parental crosses confirmed with Indel-based markers, crossing efficiency has been presented with respect to individuals tested in the laboratory.

Cross Type	Ref#	Cross Name	No. of true hybrids	Total number of seed	Crossing Efficiency (%)	Crossing Date
Chenopodium quinoa × *Chenopodium giganteum*	A14	D-12406 × Ames-19046	1	20	5	03/02/2021
	C-18*	ICBA-Q3 × Ames-19046	1	15	7	03/01/2021
	A3	Chen-254 × Ames-19046	2	13	15	03/08/2021
	A2	D-12377 × Ames-19046	6	23	26	03/08/2021
Chenopodium quinoa × *Chenopodium quinoa*	B-11	D-12401 × ICBA-Q3	1	10	10	14/02/2021
	AC-5	ICBA-Q5 × D-12377	2	11	18	04/07/2021
	C-4	ICBA-Q5 × ICBA-Q3	1	4	25	24/02/2021
	AC-8	BO-51 × D-12377	3	12	25	04/11/2021
	B-37	D-12377 × ICBA-Q5	2	5	40	03/14/2021
	B-42	D-12406 × ICBA-Q5	2	5	40	03/15/2021
	A8	D-12377 × Chen-281	11	24	46	04/07/2021
	C-9	Titicaca × D-12377	1	2	50	02/08/2021
	B-44	D-12406 × ICBA-Q3	9	17	53	03/01/2021
	B-25	D-12401 × ICBA-Q5	8	12	67	04/14/2021
	B-24	D-12401 × BO-51	15	21	71	04/14/2021

Among five parents used for natural crossing, only D12377 was black seeded parent, and this trait was used for determining the efficiency of natural crossing. There were total of 10 pots each having five parents for natural crossing purpose including D12377 as one of them. All the D12377 plants (i.e. 10 plants, one from each pot) were harvested and seed was bulked. A total of 1000 seed were sown to get 823 plants from naturally crossed black seeds (produced with D12377 as female parent). Among 823 plants, 205 and 618 produced white and black seeds respectively. As mentioned above, the 1000 seeds sown from 10 different supposedly, naturally crossed D12377 plants (i.e. we are not sure which of them underwent natural crossing). In way to analyse the crossing efficiency, we here assume that the 618 plants producing black seeds came from selfed black seeded D12377 and vice-versa for the rest 205 white seed producing plants. Therefore, with this assumption (618 black seeded plants are from selfed D12377 parent), we can inference that 205/618 × 100 ~ 25% would be the minimum crossing efficiency. It is important to note here that this efficiency was achieved under a specific situation i.e. with five plants sown in a pot and kept together in very closed vicinity and 618 black seeded plants were assumed to be derived from selfed plants (Fig. 4).

Manual observation based on distinctive diagnostic characteristics of the five parents subjected to forced flowering synchronization, crossing efficiency was observed in range from 1.9% in D12406 (5/263) to 7.7% in ICBA-Q5 (6/78). In D12401 (16/410) and D12377 (6/154) efficiency was approximately 4% (data not presented).

3.4. Inter-specific cross compatibility

Among four inter-specific crosses attempted in our study with *Chenopodium giganteum* (Ames 19046) as common male parent and Chen254, D12406, D12377 and ICBA-Q3 as females (Supplementary Figs. 3a-b). F_1 seeds of one cross (i.e. Ames 19046 × ICBA-Q3) did not produced F_2 seeds (Supplementary Fig. 3c). The other three crosses, Ames 19046 × Chen254, Ames 19046 × D12406 and Ames 19046 × D12377 successfully produced F_2 seeds.

3.5. Morphological marker identification

With respect to arrangement of hermaphrodite and female flowers, two variants were observed that could serve as morphological markers. The low saponin parent D12401 was identified most suitable for crossing because of its pink coloured hermaphrodite flowers which were clearly distinct from the female ones (Fig. 5). Similarly, in one of the high saponin line D12047, hermaphrodite and female flowers were arranged in somewhat conical fashion, in which hermaphrodite flower was placed on the tip and female ones on side (Supplementary Fig. 4).

F_1 derived from cross of D12377 × ICBA-Q5 was identified as a distinctive diagnostic panicle colour as illustrated in (Supplementary Fig. 5). In the late maturity stage, panicle colour of D12377, ICBA-Q5 and F_1 were clearly distinct. Similarly, in the natural crossing scheme, five characteristically distinct genotypes (Co 407, D12401, D12406, D12377 and ICBA-Q5), were harvested and individually bulked.

Characteristics of D12377 are green panicle and black seed colour. Another parent Co 407 has purple coloured panicle which produced white seeds. Therefore, if a F_1 plant derived from the cross of these two accessions might inherit at least one trait from each parent - D12377 and Co 407. Figure 6 presents a panicle of F1 plant in which the panicle colour is purple (similar to C0 407) producing black seed (trait from D12377). This indicates that the F_1 was produced from pollination of female flower of Co 407 by hermaphrodite flowers of D12377 or vice-versa.

3.6. Seed colour observations in quinoa

F_1 seeds derived from cross of five white seed (female) with one black seed parent (male), were all white i.e. female parent type. The F_2 seeds produced on all these five F_1s were found black coloured. Figure 7 presents the seed colour of parents (Titicaca, ICBA-Q5, ICBA-Q3, BO 51 and D12377) and F_2 seeds. Seeds of fifth cross of Chen281 (white seeded) × D12377 showed similar pattern (less seeds were produced and advanced immediately, picture couldn't be taken). These results indicated toward dominance of the black seed colour over white in quinoa. However, to better understand this pattern reciprocal crosses were made using white seeded female (ICBA-Q5) with black seeded male (D12377) and vice-versa. In both cases F_1 seeds tend to be maternal type which clearly revealed maternal inheritance, not the dominance of any seed coat colour (Fig. 8). Therefore, existence of white coloured F_2 seeds on F_1 plants made from the cross of white seeded male (ICBA-Q5) with black seeded female (D12377) rules out any conclusion on dominance of seed colour in quinoa.

Further, 76 F_2 seeds of each of the two populations Titicaca × D12377 and Chen281 × D12377 were advanced and colour of F_3 seeds were observed to better understand segregation pattern. The 76 F_2 plants of Titicaca × D12377 produced 53 black and 23 white seeds in the ratio of 2.3:1. Similarly, 76 F_2 plants of Chen281 × D12377 produced 51 black and 25 white seeds in the ratio of 2.07:1. Chi squared test performed in both crosses ruled out the significance of Mendelian monohybrid ratio of 5:3 in F3 generation indicating lack of complete dominance of black seed colour in quinoa (Table 3).

Table 3
Table presenting the chi-squared test to test “the goodness of fit” with the null hypothesis (H_0): Ratio of Black and White seeds in F3 generation were 5:3. The $F_{2:3}$ seeds were produced by two populations i.e. ‘Titicaca × D-12377’ and ‘Chen 281 × D-12377’

Titicaca × D-12377	**Black**	**White**	**Total**		
	53	**23**	**76**		
Chen 281 × D-12377	51	25	76		
Chi-Square calculation					
S.N.	Observed (O)	Expected (E)	O-E	$(O-E)^2$	$(O-E)^2/E$
1	53	47.5	5.5	30.25	0.63684
2	23	28.5	-5.5	30.25	1.0614
3	51	47.5	3.5	12.25	0.25789
4	25	28.5	-3.5	12.25	0.42982
Chi-Squared value					2.38

The chi-Squared value at α = 0.05 with degree of freedom = 1, the chi-squared value was 3.841. The calculated chi-Squared value was 2.38 which was lesser than 3.841 with 5% significance level. Therefore, we can reject the null hypothesis.

4. Discussion

Importance of quinoa outside its domestication centre i.e. Andes with ensured irrigation has been recognised in past few years. Quinoa can also be introduced/ scaled up in different parts of Asia and Africa by transforming the existing cropping systems and developing suitable varieties that can perform well under specific environments. Quinoa breeding work to develop the improved varieties is being undertaken at ICBA, UAE successfully.

4.1. Inter-specific crosses in *Chenopodium species*: Opportunity of exploiting variation

Defining a crossing block of diverse genotypes is the first step in establishing a breeding program. Low saponin, early maturity and high yield were selected as the three most important traits required for the adaptation of quinoa in newer environments. Thirteen parents in crossing blocks comprised of six *Chenopodum quinoa* and one *Chenopodum giganteum* accessions with saponin free/ very low saponin content along with six other high yielding varieties/ genotypes (Supplementary Figs. 1a-b). Diversity pattern of these 13 genotypes (Fig. 2) based on similarity index could be the best way to choose

potential parents. Interestingly, F_1 plants from three interspecific crosses made with 'saponin free *Chenopodum giganteum*' with 'saponin free *Chenopodum quinoa*' successfully produced F_2 seeds, whereas F_1 of 'saponin free *Chenopodum giganteum*' × 'high saponin *Chenopodum quinoa*' did not produced F_2 seeds.

Noticeably, the plant type of F_1s developed from inter-specific crosses (*Chenopodum quinoa* × *Chenopodum giganteum*) resembled respective *Chenopodum quinoa* parent (D12406, D12377 and Chen254), and successfully produced the F_2 seeds. Conversely, plant type of F_1 developed from ICBA-Q3 (*Chenopodum quinoa*) × Ames 19046 (*Chenopodum giganteum*) resembled *Chenopodum giganteum* parent (Ames 19046) and did not produce the F_2 seeds (Supplementary Fig. 3c). These observations clearly suggest that inter-specific crosses with *Chenopodum quinoa* with other *Chenopodum spp.* can be exploited for the desired genetic advance. The inter-specific cross compatibility and heterosis analyses can be performed at a large scale for identification of suitable genetic variants. In order to ensure adaptability of *Chenopodum quinoa* in newer environments, genetic variations created from inter-specific crosses can play a major role.

4.2. Role of distinctive phenotypic traits as diagnostic markers in natural crossing

Among 13 crossing block accessions, two distinctive diagnostic floral arrangements were observed that can greatly help in the manual crossing process. The pink-coloured hermaphrodite flowers with green-coloured female ones in quinoa genotype D12401 (Fig. 5) and the positioning of hermaphrodite flowers on the top of a cone, while female ones on sides in D12047 (Supplementary Fig. 4) render them very easily discriminated from female flowers. These flowers can, therefore, be easily removed or destroyed and female ones can be pollinated with other accessions.

The black seed colour was observed as another diagnostic characteristic that can be potentially used in natural crossing. Five quinoa genotypes underwent natural crossing (or out-crossing), as presented in Fig. 1 and were harvested individually and grown in separate plots in the next generation i.e. one genotype in one plot. True hybrids were identified successfully with the help of seed colour of F_1 seeds. If black-seeded plants produced white seeds or vice-versa were declared as true hybrids. Similarly, the purple panicle colour of Co 407 was used as a diagnostic characteristic while crossing quinoa genotypes naturally. However, in every breeding program, hybridity testing using polymorphic markers is always advisable to employ as fail-safe technology.

The probability of identifying the desired genetic variant increases by enhancing the number of crosses and population sizes. Therefore, a feasible crossing methodology ensuring a large number of crosses with minimal effort should help quinoa breeding greatly. The efficiency of three different crossing methods indicated the importance of quinoa germplasms with distinctive diagnostic characteristics, such as black seed colour and purple panicle. It was very interesting to note here that the outcrossing efficiency of the black-seeded quinoa line, D12377 was observed to be 25%, assuming that all other

black seeds were selfed. Out of 823 putative F_1 plants taking D12377 as female parent (black seeded), 205 produced white seeds and rest 618 yielded black seeds (Fig. 4). We did not observe any plant producing both black and white seed. The forced flowering synchronization method attempted in this study did not provide encouraging results, possibly because of trimming large number of flowers for synchronization. Manual crossing through removing hermaphrodite flowers, revealed a wide range of success rate indicating the role of varying floral arrangements among quinoa germplasm accessions. In nutshell, a large-scale effort in identification of distinctive diagnostic traits and floral biology in quinoa can help greatly in creating desired genetic variants that may further help in its wider adaptation in newer environments of Africa and Asia.

4.3. Marker assisted breeding: Tool that can handle crossing efficiency issue in quinoa

It is a well-known fact that due to gyno monoecious nature of flowers in quinoa, manual emasculations become difficult and less efficient. Therefore, efforts have been made in past to emasculate using different approaches including manual emasculation and hot water treatments (Emrani et al. 2020, Peterson et al. 2015, Sagar et al. 2025). However, establishing breeding pipeline and making large-scale efforts would require feasibly applicable alternatives. In this study we have presented that hybridity testing of the putative crosses through mechanical emasculation (like removal of hermaphrodite flowers) as fool proof backup strategy using abundantly available indel polymorphic markers in quinoa (Fig. 3, Table 2). Therefore, the bottleneck of low efficiency of manual crossing in quinoa can be effectively addressed by using marker assisted breeding approaches. The aforesaid methods of crossing could be used to fast-track breeding efforts in difficult crop like quinoa, in conjunction with hybridity testing using indel markers as fool-proof technology.

4.4. Black seed colour trait in quinoa – a mystery

Four intra-specific crosses were performed taking black seeded D12377 as male parent with four different white seeded female parents as shown in Fig. 7. All four crosses produced white coloured F_1 seeds. The F_1 seeds were grown and the F_1 plants produced black coloured F_2 seeds, thereby indicating toward dominance of the black seed colour (Fig. 7). Further, in order to understand the dominance/ recessive behaviour of seed colour, the black 76 F_2 seeds of two populations *viz*. Titicaca × D12377 and Chen281 × D12377 were grown to produce $F_{2:3}$ seeds. Assuming that the seed colour is the trait of seed itself, expected Mendelian ratio of black vs white seeds should be ~ 5:3. To confirm this, chi-squared test was performed which ruled out the significance of Mendelian 5:3 ratio (Table 3). Therefore, the possibility of dominance of black seed colour in quinoa germplasm materials investigated in this study was ruled out in this study. However, more robust and large-scale studies may help for an improved understanding of the dominance/recessive nature of black seed colour in quinoa.

Further, in another experiment reciprocal crosses with white and black-seeded quinoa genotypes were made to better understand the black and white seed colour inheritance. Interestingly, these results

revealed maternal and paternal seed colour trait expressions in F_1 and F_2 seeds respectively as presented in Fig. 8. The paternal seed colour expression in F_2 seeds might be attributed to the pollen mediated gene transfer/expression/interaction in quinoa. The evidences generated in current study were inconclusive. Therefore, in conclusion, further in-depth genetic, cytological and molecular analyses are required to better understand the seed colour inheritance in quinoa.

4.5. Genetic improvement of quinoa with improved breeding methods and approaches

Our study revealed the suitability of quinoa crossing approaches for diverse germplasms harbouring an array of floral diversity. In this study we have presented distinctive diagnostic traits that can help in improving efficiency of quinoa breeding, specifically differentiating characters of hermaphrodite flowers in some quinoa accessions. These distinctive traits enabled visual/ morphological identification of the true hybrids. In addition, we have observed the effectiveness of combined approach including both diagnostic and molecular markers in hybridity testing and genetic analysis that are important pre-requisites of a 'breeding program'. The distinctive traits and floral arrangements of some accessions were so effective, that they facilitated "deliberate natural out-crossing" in quinoa, for example the black seed colour of accession, "D12377" or purple panicle colour of "Co 407". Our findings suggest that large-scale identification of distinctive diagnostic characteristics in quinoa is urgently required.

A feasible quinoa breeding scheme has been presented in Fig. 9. The success of the proposed breeding scheme underlies parental stock with distinctive diagnostic characteristics, for example, black seed, purple panicle, coloured hermaphrodite flowers etc. Secondly, the cross ability due to floral size and shape of parental lines is a critical parameter which significantly affects the natural crossing. The parental stock lines with distinctive diagnostic characteristics could be planned in a specific arrangement to facilitate natural crossing followed by selection of putative F1s based on key adaptive traits such as vigor, color, grain yield or earliness. Thereafter, generation advance can be made by breeding selections for preferred traits in every generation till sufficient homozygosity is attained. These fixed lines can then be subjected to multilocation yield stability trails as part of varietal pipeline. During this process, fixed genotypes with unique diagnostic characters can be identified and added to the parental stock for the next cycle of breeding advancements. Similar breeding schemes can be formulated based on identified distinctive diagnostic characteristics and pursued for breeding suitable varieties. These efforts are crucial for the global expansion of quinoa, particularly in newer environments of Asia and Africas.

5. Conclusions

The complex floral morphology of quinoa is the most important bottleneck in crossing and thereby its genetic improvement, which ultimately renders limited varietal diversity for varying crop ecologies in different parts of the world. The study presents the importance of the characterization of germplasm resources for the identification of the distinctive diagnostic traits that can be further utilized in

overcoming crossing barriers. The study also validates the importance of ‘indel’ markers in quinoa breeding, particularly the marker-assisted hybridity testing. In this research, an interesting pattern was observed related to the black seed color of quinoa, which requires further in-depth analysis to better understand the genetics behind it. In a nutshell, quinoa improvement for newer environments can be followed in a sequential manner starting from crossing block definition, distinctive diagnostic trait identification for overcoming crossing barriers to marker-assisted hybridity testing, and breeding selection for ecology-specific desired traits.

Declarations

Acknowledgments

The authors hereby duly acknowledge funding support provided by the International Center for Biosaline Agriculture, UAE. Authors are also thankful to all intellectual and non-technical inputs provided by researchers at ICBA and other institutions.

Author contributions

PV, RKS and HR designed experiments; PV and SVJ were responsible for crossing, PV, LM, HR and SA performed hybridity testing; SNR and MGK were responsible for data gathering; SS, PV, GPM and RKS interpreted the results; PV drafted the manuscript. All authors contributed to the article and approved the submitted version.

Data availability statement

The original contributions presented in the study are included in the article/Additional Material. Further inquiries can be directed to the corresponding author.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This research was conducted by institutional research fund of the International Center for Biosaline Agriculture, UAE.

Ethics approval and consent to participate

Not applicable.

References

1. Jacobsen SE (1997) Adaptation of quinoa (*Chenopodium quinoa*) to Northern European agriculture: studies on developmental pattern. Euphytica 96:41–48
2. Jacobsen SE (2003) The worldwide potential of quinoa (*Chenopodium quinoa* Willd.). Food Rev Int 19:167–177
3. Jacobsen SE (2015) Adaptation and scope for quinoa in northern latitudes of Europe. In: FAO, CIRAD (eds) State of the art report on quinoa around the world in 2013. FAO, Rome, pp 436–446
4. Amin QA, Wani TA, Nahvi AI (2022) Quinoa is a gluten-free, high-nutrient and underutilized crop with a lot of processing possibilities. Agric Food E-Newsl 2:3
5. Anderson JA, Churchill GA, Autrique JE, Tanksley SD, Sorrells ME (1993) Optimizing parental selection for genetic linkage maps. Genome 36:181–186
6. Bazile D, Jacobsen SE, Verniau A (2016) The global expansion of quinoa: trends and limits. Front Plant Sci 7:622. https://doi.org/10.3389/fpls.2016.00622
7. Collard BCY, Mackill DJ (2009) Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol Biol Rep 27:86–93
8. Cota-Sanchez JH, Remarchuk K, Ubayasena K (2006) Ready-to-use DNA extracted with a CTAB method adapted for herbarium specimens and mucilaginous plant tissue. Plant Mol Biol Rep 24:161–167
9. Emrani N, Hasler M, Patiranage DSR, Maldonado N, Rey E, Jung C (2020) An efficient method to produce segregating populations in quinoa (*Chenopodium quinoa*). Plant Breed 139:1190–1200. https://doi.org/10.1111/pbr.12858
10. Fleming JE, Galwey NW (1995) Quinoa (*Chenopodium quinoa*). In: Williams JT (ed) Cereals and pseudocereals. Chapman and Hall, London, Underutilized Crops Series, vol 2, pp 3–83
11. James LEA (2009) Quinoa (*Chenopodium quinoa* Willd.): composition, chemistry, nutritional, and functional properties. Adv Food Nutr Res 58:1–31
12. Koziol MJ (1991) Afrosimetric estimation of threshold saponin concentration for bitterness in quinoa (*Chenopodium quinoa* Willd.). J Sci Food Agric 54:211–219
13. Murphy KM, Bazile D, Kellogg J, Rahmanian M (2016) Development of a worldwide consortium on evolutionary participatory breeding in quinoa. Front Plant Sci 7:608. https://doi.org/10.3389/fpls.2016.00608
14. Patirange DS, Rey E, Emrani N, Wellman G, Schmid K, Schmöckel SM et al (2022) Genome-wide association study in the pseudocereal quinoa reveals selection pattern typical for crops with a short breeding history. Elife 11:e66873. https://doi.org/10.7554/eLife.66873
15. Peterson A, Jacobsen SE, Bonifacio A, Murphy K (2015) A crossing method for quinoa. Sustainability 7:3230–3243. https://doi.org/10.3390/su7033230
16. Rahman H, Vikram P, Hu Y, Asthana S, Tanaji A, Suryanarayanan P et al (2024) Mining genomic regions associated with agronomic and biochemical traits in quinoa through GWAS. Sci Rep 14:19205. https://doi.org/10.1038/s41598-024-69992-2

17. Sagar P, Chatterjee S, Joshi DC, Tiwari JK (2025) Standardization of different techniques for the rapid production of F1 hybrids in *Chenopodium quinoa*. Euphytica 221:123. https://doi.org/10.1007/s10681-024-03456-7
18. Sha X (2013) Rice artificial hybridization for genetic analysis. Methods Mol Biol 956:1–12
19. Simmonds N (1971) The breeding system of *Chenopodium quinoa* I. Male sterility. Heredity 27:73–82
20. Singh D (2019) Quinoa (*Chenopodium quinoa* Willd.). Scientific Publishers, Jodhpur
21. Tabatabaei I, Alseekh S, Shahid M, Leniak E, Wagner M, Mahmoudi H et al (2022) The diversity of quinoa morphological traits and seed metabolic composition. Sci Data 9:323. https://doi.org/10.1038/s41597-022-01435-8
22. Ward S, Johnson D (1994) A recessive gene determining male sterility in quinoa. J Hered 85:231–233
23. Zhang T, Gu M, Liu Y, Lv Y, Zhou L et al (2017) Development of novel InDel markers and genetic diversity in *Chenopodium quinoa* through whole-genome re-sequencing. BMC Genomics 18:685. https://doi.org/10.1186/s12864-017-4003-2
24. Zurita-Silva A, Fuentes F, Zamora P, Jacobsen SE, Schwember AR (2014) Breeding quinoa (*Chenopodium quinoa* Willd.): potential and perspectives. Mol Breed 34:13–30

Figures

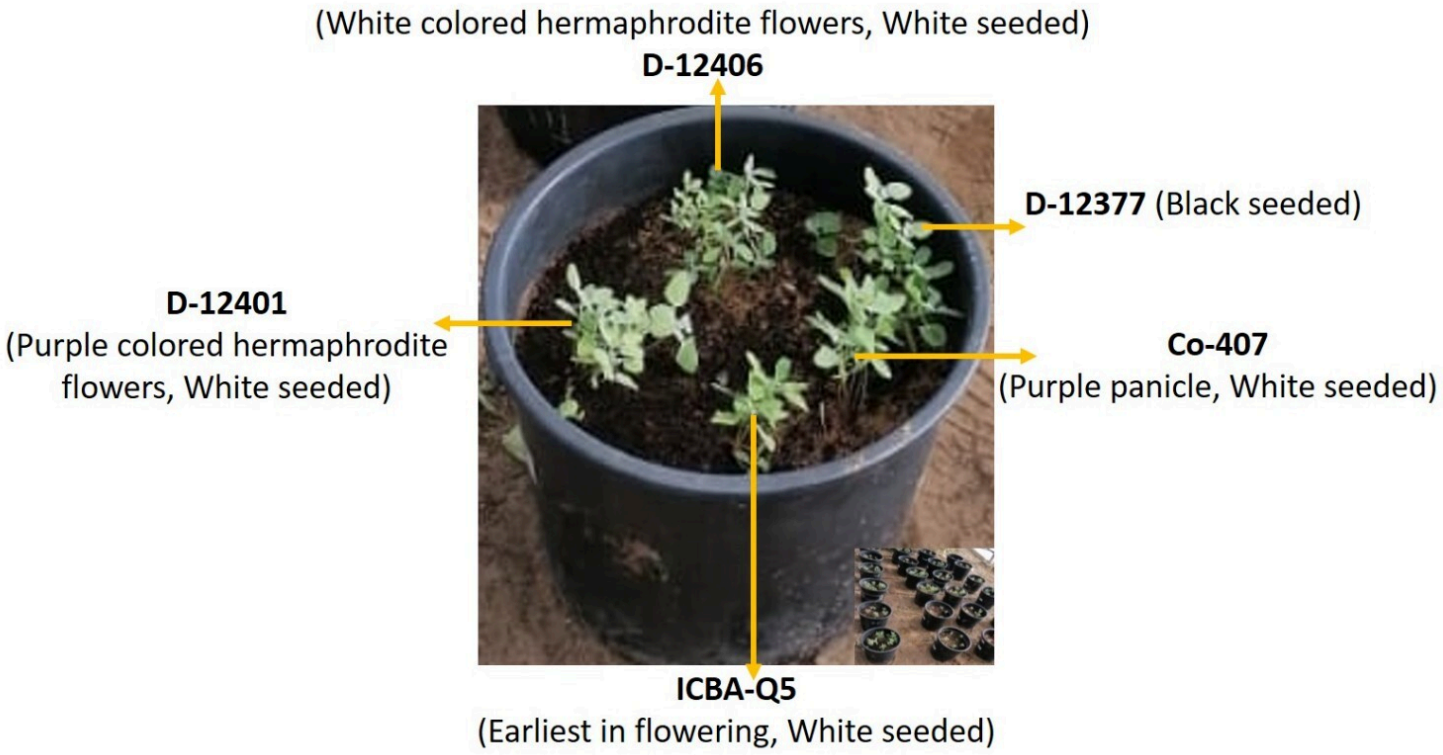

Figure 1

Figure presenting the arrangement of five different quinoa genotypes with distinctive diagnostic characteristics. Orientation of all the five plants was kept same in all pots.

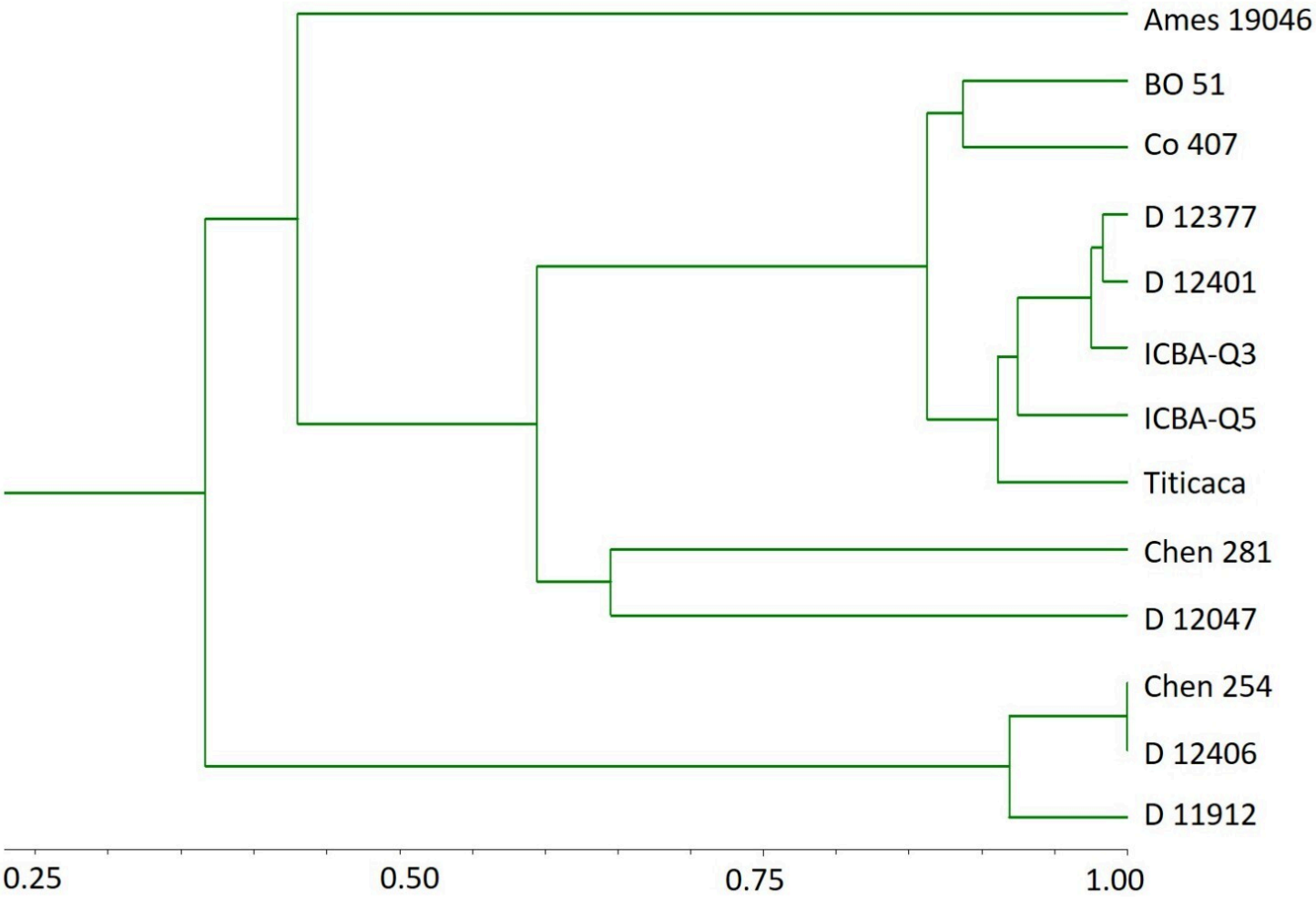

Figure 2

Dendrogram presenting diversity pattern of the crossing block accessions.

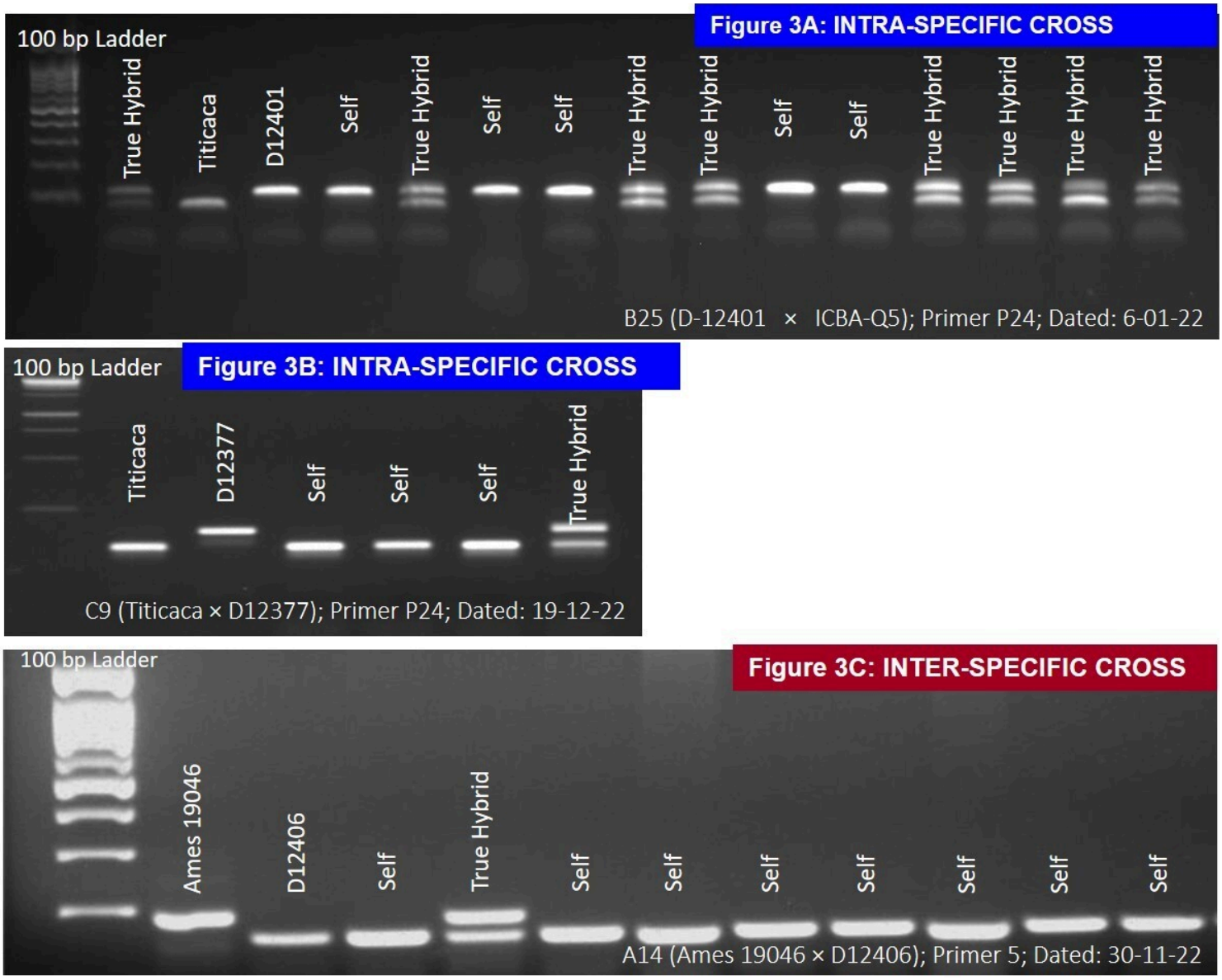

Figure 3

(A): Hybridity testing of the cross between well adapted variety, 'Titicaca' and saponin free line 'D12401'; **(B):** Hybridity testing of the cross between well adapted variety, 'Titicaca' and saponin free black seeded line 'D12377'; **(C):** Hybridity testing of the cross between Chenopodium giganteum (Ames 19046) and Chenopodium quinoa (D12406)

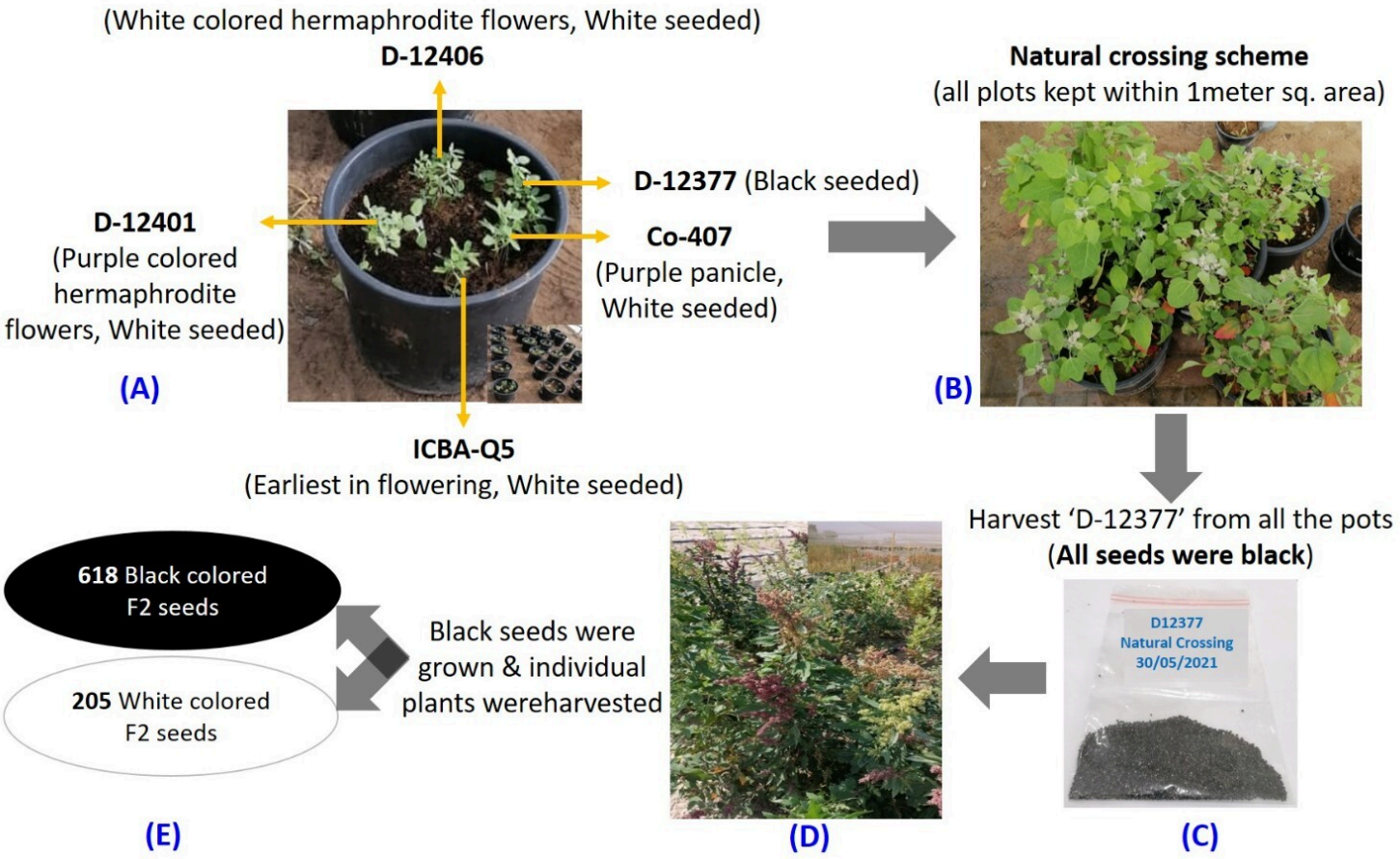

Figure 4

Figure presenting the natural crossing scheme using black seeded quinoa enabling hybrid identification through seed colour of progenies

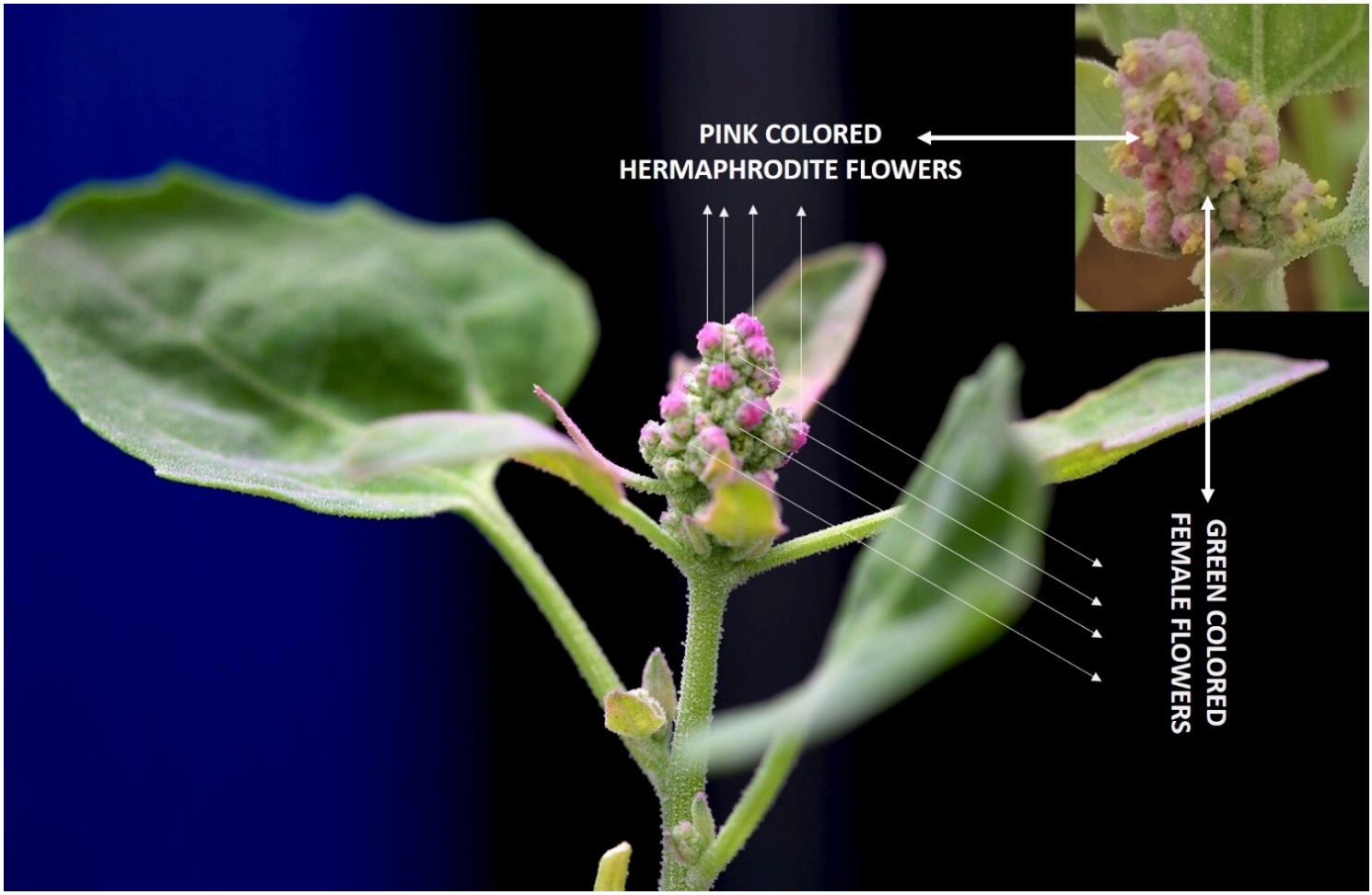

Figure 5

Pink colored hermaphrodite flowers identified in quinoa accession- D12401

Figure 6

Figure presenting morphological identification of true crosses in quinoa having diagnostic characteristics. Among 5 parents that underwent natural crossing, Co 407 was white seeded - purple panicle plant, whereas D-12377 was black seeded –green panicle plant. The naturally crossed panicle presented here had purple panicle harbouring black seed confirming cross between Co 407 and D12377. Yellow arrows pointing the black seed formation

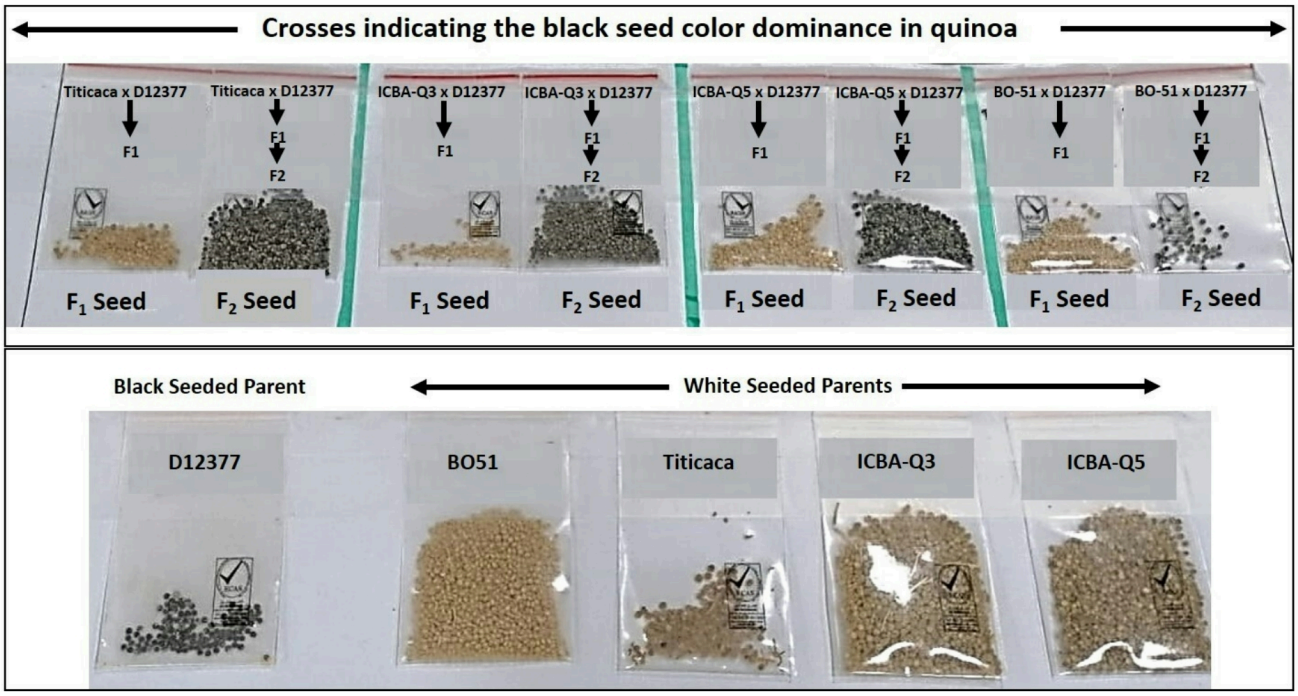

Figure 7

Figure presenting the dominance of black seed colour in quinoa. Black seeded parent was crossed with four different white seeded genotypes and F2 from all four crosses were black.

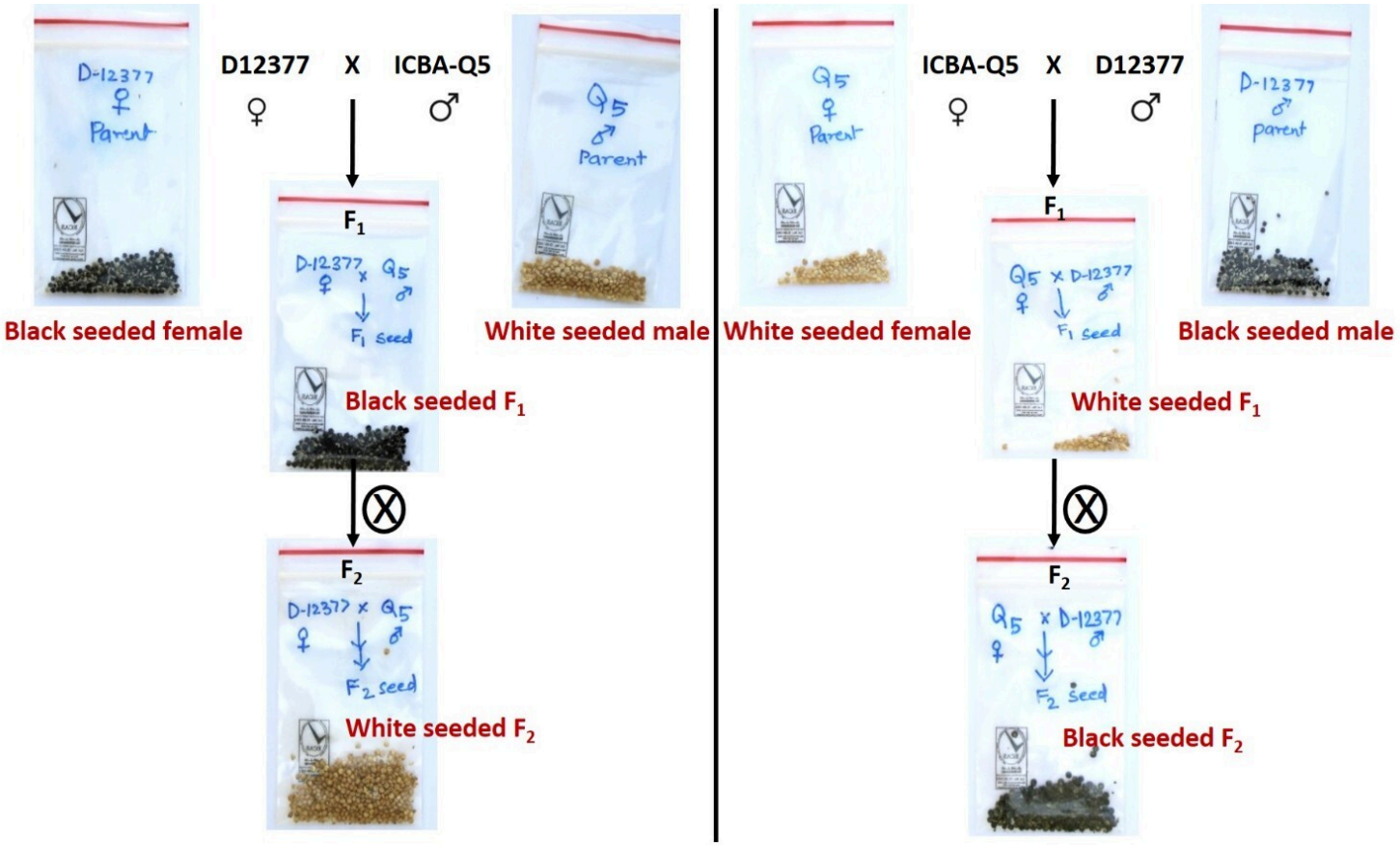

Figure 8

Figure presenting the black – white seed colour variation in reciprocal crosses of quinoa

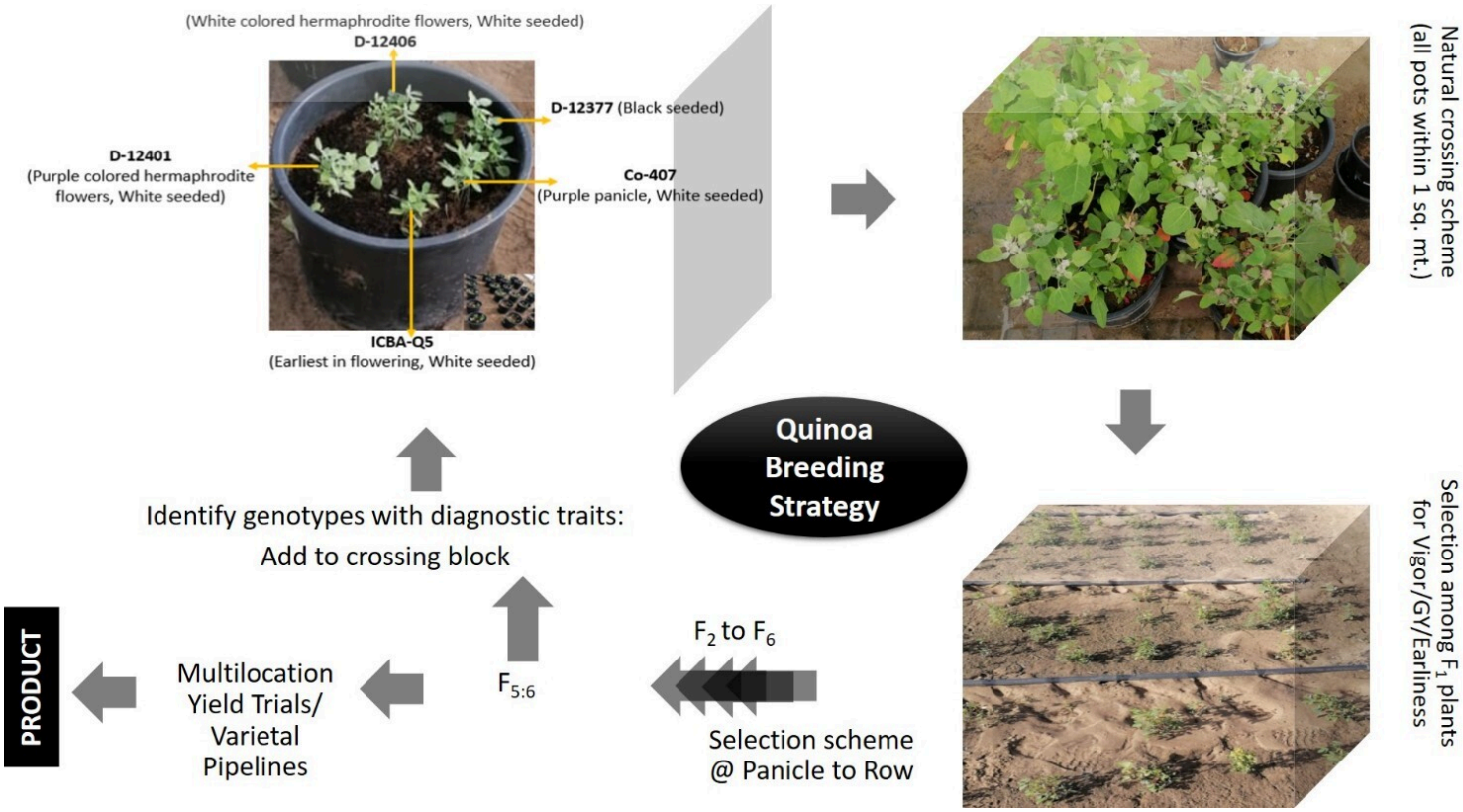

Figure 9

Flow chart proposing a fast-track quinoa breeding pipeline strategy through identifying and utilizing parental stocks with diagnostic characteristics

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.pdf