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# Strategic genetic amelioration of quality protein maize (QPM) germplasm and its utilization in hybrid breeding

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## Abstract

Use of diverse germplasm for generating heterotic hybrids is the foremost requirement in maize. The present study was conducted by using a diverse set of inbred lines and the line × tester method was applied to identify best performing lines and to group QPM inbred lines into different heterotic groups. The test crosses, developed by following line (66) × tester (CML 161 and CML 165) mating design, were evaluated during *winter* 2013, *rainy* 2014 and 2015 seasons at Begusarai and Ludhiana, respectively. Based on the specific combining ability, the lines were categorized into two heterotic groups. Out of 66 novel inbreds, 18 lines with significant SCA with CML165 were classified in group A, 16 inbreds with significant SCA with CML161 were classified in group B and 20 inbreds with significant GCA were classified in group (AB). Nine inbred lines were selected based on their positive GCA values and pedigree crosses were developed in *rainy* season in 2017. Three crosses were made in heterotic group A and four crosses were in group B for synthesizing new inbred lines by using pedigree method. Heterotic grouping based inbred evaluation trial and biochemical analysis were carried out to estimate *per se* yield potential of developed lines and to estimate tryptophan content. QIL-4-2491 (Group-A) and QIL-4-2401 (Group-B) were the top yielders. A total of 25 crosses were made among the heterotic groups (A and B) by using 22 lines from groups A and B and three best performing hybrids were identified.

## Introduction

Maize (*Zea mays* ssp. *mays* L.) is an important crop cultivated across the world and contributing approximately 35.7% to the total worldwide cereal production. Maize is a major source of food for human consumption and livestock production. It is also used as a raw material for many agro-allied industries in the world (Undie *et al.*, 2012; Sharma *et al.*, 2023). Over half of the global maize production (54.5%) is together produced by USA and the China *i.e.* 361 and 259 M t/pa, respectively (Erenstein *et al.*, 2022; Kaur *et al.*, 2022; Yathish *et al.*, 2023). All of the three big staple cereals, *viz*., wheat, rice and maize comprise a major part of the human diet, on the other hand, it also accounts for estimated 42% of the world's food calories and 37% of protein intake (average 2016–18, FAO STAT, 2021). Thus, in order to fulfil population demands as well as meeting the goals for food and nutritional security at the global level, the role of the maize is highly diverse as well as dynamic in agri-food systems (Grote *et al.*, 2021).

Despite contributing around 15% to the global protein consumption, maize proteins have a low nutritional value (Nuss and Tanumihardjo, 2010). Though normal maize kernel contains 8–11% protein in endosperm, but is inherently deficient in two essential amino acids namely lysine and tryptophan (which the human body can't synthesize and must obtain from food) (Vasal, 1999). In normal maize varieties, the lysine and tryptophan content is less than half of the recommended rate for human nutrition (FAO/WHO-Expert consultation, 1990). In Central and South America, Africa and Asia, majority of population consume maize as their staple food, for weaning babies, and for feeding livestock. So, it typically causes malnutrition unless it is consumed along with other food sources which the majority of people cannot afford in the developing regions. Babies weaned on it are frequently underweight, prone to diseases, at high risk for starvation and have malnutrition disorders such as marasmus, kwashiorkor and pellagra. Pellagra is basically comprised of three main diseases viz. diarrhoea, dermatitis and dementia (Hegyi *et al.*, 2004).

In the 1920s, a naturally occurring mutant of maize, *opaque-2*, was found in the USA that had soft endosperm (Vietmeyer, 2000). In 1961, it was found that homozygous *o2* maize has higher levels of lysine and tryptophan (Mertz *et al.*, 1964). These two amino acids allow the body to digest complete proteins; thereby eliminating wet-malnutrition (Mamatha *et al.*, 2017). During the early 1970s, Villegas and Dr Surinder K. Vasal began their collaborative research

on developing QPM varieties in Mexico. However, *opaque-2* had lower yields and a soft, chalky kernel which made it more susceptible to ear rot and insect damage.

One of the finest outcomes of the several efforts directed towards the improvement of protein quality in maize, at CIMMYT in the late 1990′s, was the hard endosperm *o2* genotypes, most commonly referred to as Quality Protein Maize (QPM) (Vasal, 2000). The addition of QPM in the diet improved health of babies and adults by lowering the risk for malnutrition disorders such as marasmus and kwashiorkor. Pigs fed on QPM showed rapid weight gain and thus provide an additional quality protein source for small farm families (www.worldfoodprize.org).

The determination of heterotic patterns of the available germplasm is necessary for the success of breeding programmes in maize (Kumar *et al.*, 2022; Karjagi *et al.*, 2023). The constitution of heterotic groups acts as foundation for maize breeding. Furthermore, classification of heterotic groups is essential in order to improve breeding efficiency (Das *et al.*, 2021). By classifying the maize inbreds into known heterotic groups, numbers of duplicates can also be reduced and at the same time, diversity can also be maintained. It is commonly assumed that the combination of lines of different heterotic groups results in higher expression of the target trait in hybrids contrary to the hybrids from same group or origin (Ricci *et al.*, 2007). In the past, factor that had contributed critically to the success of single-cross hybrid maize breeding is the classification of elite germplasm into heterotic groups. Development of heterotic group is one of the most major step in maize hybrid breeding programme which can help to save the natural resources such as water, soil, minerals etc. by avoiding the evaluation of all the possible crosses emerging from the sets of inbred lines. Several methods were used by different researchers to assess heterosis and to categorize the germplasm into different heterotic groups. In maize breeding programmes, combining ability analysis is largely used to assess general combining ability (GCA) of lines as well as specific combining ability (SCA) of hybrids to have information about gene actions involved, diversity evaluation as well as for hybrid development and heterosis estimation. The major models used in combining ability analyses are the diallel mating models developed by Griffing (1956) and Gardner and Eberhart (1966). Most predominant models were the conventional crossing designs such as line × tester, partial diallel, three-way crosses and molecular based studies. Combining ability could be defined as the potential of inbreds to hybridize so that only desirable alleles could pass on to next generation. Griffing (1956) stated that GCA is the average performance of a parent in a series of hybrid combinations, whereas SCA is the difference in performance of certain hybrid combinations as contrary to the results or relations that would be expected as based on the GCA. GCA is regarded as additive gene effects while SCA reflects the non-additive gene actions (Sprague and Tatum, 1942). Fan *et al.* (2008) proposed the heterotic groups specific and general combining ability (HSGCA) method for considering both GCA and SCA to classify the lines into the clear-cut heterotic groups. In a recent study conducted by Arora *et al.* (2024), the heterotic grouping of 78 yellow maize inbred lines was done using combining ability as well as molecular diversity using single nucleotide polymorphisms (SNPs) markers which facilitated the grouping of 18 inbred lines in HG-A and 33 inbred lines in HG-B.

The HSGCA method was used by several researchers (Akinwale *et al.*, 2014; Badu-Apraku *et al.*, 2015; Amegbor *et al.*, 2017; Olayiwola, 2018) who found that it is more efficient than the SCA method and even marker-based methods. In literature, it has been reported that some of the researchers conducted studies to evaluate both methods of analysis *i.e.* the SCA and HSGCA (Fan *et al.*, 2009; Badu-Apraku *et al.*, 2015; Chemeli, 2016; Singode *et al.*, 2017; Arifin *et al.*, 2018). The number of heterotic groups being used significantly affects the ratio of the number of varieties adopted or released as well as cost-benefit ratio. Also, heterotic grouping improves the identification of viable commercial hybrids and then as a result, pre-hybrid cost will be reduced (Ceccarelli, 2015). To systematically exploit the effect of heterosis in maize, the classification of genotypes into genetically divergent heterotic groups have always been suggested. Therefore, the present study was conducted to identify superior novel QPM inbreds and to generate information on heterotic pattern of newly developed QPM inbred lines for better utilization in future QPM hybrid breeding programmes.

## Materials and methods

### Development of new QPM inbred lines

Novel QPM inbreds were derived from different hybrids of public and private origin, variants of tropical inbreds including CIMMYT maize lines (CMLs), and also from pools and populations (online Supplementary Table S1). Inbred lines were derived after six generations of continuous self-pollination and selection in the fields of Indian Institute of Maize Research (IIMR), Ludhiana.

### Generation of test crosses and their heterotic grouping

Two inbreds, *viz.*, CML 161 and CML 165 collected from CIMMYT, Mexico, were used as testers to classify these inbreds into distinct heterotic groups. Different set of inbreds were crossed with the same testers (CML 161 and CML 165) following line × tester mating design in three consecutive seasons, *viz.*, *rainy* 2013, *winter* 2013 and *winter* 2014. The newly developed inbred lines were used in every season as soon as the inbreds achieved the desired level of purity. During *rainy*, 2013, 15 lines were used at Delhi to generate 30 crosses, whereas 32 and 19 lines were used during *winter,* 2013 and *winter*, 2014 to generate 64 and 38 crosses respectively at Begusarai. Hence in total, 132 test crosses were generated following line × tester mating design by crossing 66 newly developed QPM inbred lines with two inbred testers. Synchronization of flowering among the testers and inbred lines was achieved by staggered planting of testers at an interval of 7 d during *rainy* and 15 d during *winter*.

### Evaluation of testcrosses

Testcrosses generated during *rainy* 2013 were evaluated during *winter* 2013 at Begusarai whereas crosses generated during *winter* 2013 and *winter* 2014 were evaluated at Ludhiana during *rainy* 2014 and 2015, respectively. Trials were conducted using a randomized complete block design (RBD). Hybrids were grown in three replications each having 3 m row length, 75 cm row-to-row and 20 cm plant-to-plant distance. Standard agronomic practices were followed to raise a good crop during all three seasons. The grain yield was recorded at 15% moisture content.

### Selection of inbred lines based on combining ability and development of pedigree crosses

GCA of different inbred lines was calculated and nine inbred lines were selected based on their positive GCA Values. The pedigree


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sources/origin of these selected diverse inbred lines is listed in online Supplementary Table S1. The inbred lines within the group A was crossed with each other and similarly same crossing pattern was followed in group B, to form the seven diverse pedigree crosses in *rainy* season, 2017 at ICAR-IIMR farm, Ludhiana. The pedigree crosses generated from the crosses between inbred lines of group A was heterotic grouped in Group A. Similarly, the pedigree crosses which were heterotic grouped in Group B, were developed by making the crosses between inbred lines of group B.

### Development of novel QPM lines/progenies from pedigree crosses

The progenies of all the seven pedigree crosses was developed through the pedigree method for the subsequent three years (2017–2020) at ICAR-IIMR Ludhiana (*rainy* and *spring* season) and at RMR & SPC, Begusarai (*winter* season) and a wide number of $F_2$ derived $F_3$ progenies (948) were advanced and these families were further maintained by selfing till they achieved maximum level of uniformity in $F_8$ generation. The light board/table was used for screening of the kernels and based on the opacity, they were advanced to further generations. The pedigree method of breeding used to develop progenies consists of a number of steps over the years. In this method, individual plants were reselected from $F_2$ and subsequent generations, their progenies were grown and a record of parent-progeny relationship (pedigree) was maintained. Each progeny in the every generation can be traced back to the $F_2$ plant from which it originated.

### Light table for screening of QPM germplasm

A light table is a custom made box used to differentiate hard endosperm maize types from the soft *o2o2* genotypes. The top surface is made up of semi-transparent glass or plastic. Inside the box, there are one or more florescent bulbs or lights. This box is connected with an outside power source. Light table selection is based on the principle that *o2o2* genotypes carry an undesirable characteristic, kernel softness, which, on a light table, is seen as complete opaqueness. To view the kernel characteristics, maize kernels are placed on the table and light is turned on. The endosperms when placed on a light table do not transmit light compared with the normal wild-type kernels that are vitreous and translucent. This forms a very important step in the selection process for QPM maize.

### Evaluation of novel $F_8$ families for yield

After generation of inbred lines, *per se* performance of $F_8$ families was assessed by conducting an inbred evaluation trial in *rainy* 2020 at ICAR-IIMR farm at Ludhiana. Trial was conducted in RBD experimental design with two replications. Grain yield (q/ha) was recorded for all of the inbred lines tested in the trial.

### Development and evaluation of hybrids generated by crossing inbred lines from opposite heterotic groups

Crosses were made among the best performing inbred lines from groups A and B in *rainy* season in 2021 at IIMR, Ludhiana location. These hybrids/crosses were then evaluated for grain yield in growing season in *winter* 2021 at RMR & SPC, Begusarai. The hybrid evaluation trial was conducted in a 3 m row length with two replications in RBD. The checks were included in this trial for providing a reference to the grain yield of tested cultivars. Standard agronomic practices as well as plant protection measures were followed at each experimental site. After harvesting, grain yield q/ha was calculated at 15% moisture content.

### Statistical analysis

The grain yield data was subjected to analysis of variance (ANOVA) separately for each of the seasons. Similarly, the GCA and SCA effects for grain yield was calculated separately for each season by following the line × tester model in SAS® ver. 9.3 package, SAS Institute Inc. (2011) (www.sas.com/en_in/software/stat.html). The statistical SPSS software, IBM Corp. (2021) (www.ibm.com/products/spss-statistics) was used for carrying out the ANOVA separately for the grain yield of inbred lines tested in inbred evaluation trial (progenies of pedigree crosses) as well as hybrids developed through crosses between lines belonging to different heterotic groups.

### Tryptophan estimation

The tryptophan content (% Trp per 100 g of protein) was estimated in the samples defatted for different time intervals (Hernandez and Bates, 1969). For this purpose, 100 mg of defatted maize endosperm sample was digested using 4 ml of papain solution. After incubation (65°C for 16 h), the samples are allowed to cool in order to make the supernatant clear. 1 ml of supernatant was pipetted out in a test tube and treated with 4 ml of reagent C. Reagent C was made by mixing a volume to volume mixture of reagent A (Ferric chloride six-hydrated Glacial acetic acid) and reagent B (Sulfuric acid (analytical)). The test tubes were kept in an incubator at 65°C for 15 min for colour development. The solution was transferred to calibrated tubes and the coloured complex was measured at 545 nm in a UV-Vis double beam spectrophotometer (model UV 2080) from Analytical technologies limited. All the inbred lines were tested for tryptophan content as well as half kernel weight.

## Results

### Analysis of variance of testcrosses for L × T

The ANOVA of L × T design is presented in online Supplementary Table S2. Variation among inbred parents was significant during *winter* 2013, *rainy* 2014 and *rainy* 2015 representing great diversity in the parental lines. Significant differences among testcross hybrids were observed for all three seasons. Thus, significant variability was present in the newly developed testcrosses. Effects of crosses was partitioned into lines, testers and line × tester effects. Pattern of variation of inbreds used as lines was reflected in parents. Tester effects were non-significant in all the three locations. Line × tester effect showed significant interaction between lines and testers for each season indicating hybrids differed significantly in their SCA effects.

### Variation in yield of hybrids generated in L × T design

During *winter* 2013, mean grain yield of test crosses varied from 61.67 (QIL-4-2052 × CML 161; 57.39 q/ha, QIL-4-2052 × CML 165; 65.96 q/ha) to 106 (QIL-4-2053 × CML 161; 117.83 q/ha, QIL-4-2053 × CML 165; 94.17 q/ha). Highest mean yield with both the testers was recorded in test cross involving inbred


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QIL-4-2053 followed by QIL-4-2063 and QIL-4-2047. Whereas testcross mean yield varied from 59.52 (59.52 q/ha of QIL-4-2053-1 with both testers CML 161 and CML 165) to 125.00 (QIL-4-2034 × CML 161; 126.19 and QIL-4-2034 × CML 165; 123.81 q/ha) during *rainy* 2014 and 25.47 (22.60 q/ha grain yield of QIL-4-2216 × CML 161; 28.33 q/ha grain yield of QIL-4-2216 × CML 165) to 37.25 (QIL-4-2184 × CML 161; 31.60 q/ha, QIL-4-2184 × CML 165; 42.90 q/ha) during *rainy* 2015. Some of the inbred lines had mean grain yield above >100 q/ha and thus superior yield with both the testers CML 161 and CML 165, these inbred lines were, QIL-4-2034 (125 q/ha), QIL-4-2042 (117.85 q/ha), WNC-19082 (114.285 q/ha), QIL-4-2018-1 (113.09 q/ha), QIL-4-2064 −1 (111.90 q/ha), QIL-4-2017 (107.145 q/ha) and QIL-4-2038-1 (107.145 q/ha) during *rainy* 2014. QIL-4-2184 (37.25 q/ha), QIL-4-2230 (36.86 q/ha), QIL-4-2248 (34.33 q/ha), QIL-4-2238 (35.44 q/ha), QIL-4-2192 (35.42 q/ha), QIL-4-2261 (35.25 q/ha), DML1302 (33.98 q/ha), QIL-4-2274(33.95 q/ha) and QIL-4-2172 (32.75 q/ha) recorded above mean testcross yield (>32.05 q/ha) during *rainy* 2015 (online Supplementary Table S3).

### Combining ability of lines and crosses

GCA is associated with additive and additive × additive gene effects which are largely intra allelic interactions whereas SCA is related with dominance and/or epistatic effects. Twenty three inbred lines, *viz*., QIL-4-2047, QIL-4-2053, QIL-4-2063, QIL-4-2072, QIL-4-2085, QIL-4-2065-1, WNC-19082, QIL-4-2064-1, QIL-4-2018-1, QIL-4-2042, QIL-4-2034, QIL-4-2052, QIL-4-2017, QIL-4-2038-1, WNC-18737, QIL-4-2050-1, QIL-4-2192, QIL-4-2230, QIL-4-2238, QIL-4-2211, QIL-4-2248, QIL-4-2261 and QIL-4-2184 showed positive and significant GCA effects, thus have high potential to transfer desirable traits to their progenies and could be exploited in maize improvement programs for grain yield (online Supplementary Table S3). QIL-4-2034 was identified as best general combiner with the maximum GCA effect (36.21**), whereas QIL-4-2238 and QIL-4-2261 were poor combiners with the lowest GCA effect (2.40**). Fourteen inbred lines viz., QIL-4-2057(18.47*), QIL-4-2080-1(8.17**), QIL-4-2065-1(3.64*), QIL-4-2022(4.94**), QIL-4-2053-1(4.37**), QIL-4-2039-1(12.47**), QIL-4-2077(37.24**), QIL-4-2042 (13.91**), QIL-4-2028-1(3.52*), QIL-4-2034(5.27**), QIL-4-2025(5.37**), WNC-18737(10.64**), QIL-4-2057-1(11.62**) and DML1302(7.66*) recorded significant positive SCA effects with tester CML 161 whereas ten inbred lines, *viz*., WNC10175(12.04**), WNC19082(9.36**), QIL-4-2064-1(4.06*), QIL-4-2018-1(23.10**), QIL-4-2023(18.86**), QIL-4-2026-1(18.76**), QIL-4-2052(6.13**), QIL-4-2066-1(7.43**), QIL-4-2017(5.83**) and QIL-4-2248(9.84**) revealed significant positive SCA effects with tester CML 165. QIL-4-2077 × CML161 and QIL-4-2018-1 × CML 165 recorded highest SCA effects with tester CML161 and CML165, respectively. *Rainy* 2014 recorded the highest location mean (87.39 q/ha) as compared to *winter* 2013 (75.54 q/ha) and *rainy* 2015 (32.05 q/ha), respectively and most of the lines were also grouped based on this season data.

### Classifying inbred lines into heterotic groups

The classification of maize inbreds into heterotic groups facilitates maximum exploitation of heterosis and serves as source germplasm for pools and populations from which superior second cycle inbreds could be derived. Inbreds were classified into different heterotic groups following the method of Menkir *et al.* (2004) with minor modification. Two testers, CML161 (A) and CML165 (B) were used as the bases of classification of inbreds into heterotic groups. These are the parents of released hybrid 'Shaktiman 5', are diverse lines and also reported as established testers by CIMMYT, Mexico. Inbred lines recorded significant positive SCA effects with tester CML161 (A) but having negative SCA effects with tester CML165 (B) were placed into the heterotic B group. Likewise, inbred lines exhibiting significant positive SCA effect with tester CML165 (B) but having negative SCA effects with tester CML161 (A) were placed into the heterotic A group. Lines showing significant positive GCA effects were placed in the heterotic group AB; even though they recorded significant SCA effects. Mean value of testcrosses of all the inbred lines with tester A (CML 161) was subtracted from the mean value of testcrosses of all the inbred lines with tester B (CML 165) in each season and the obtained value was used as minimum benchmark (x) for heterotic grouping. Each inbred line when crossed to tester A (CML 161) and tester B (CML 165), and the difference of yield of testcross progeny, more than 'x', was considered for heterotic grouping. However, inbred lines showing difference below 'x' were not considered for heterotic grouping. During *winter* 2013 and *rainy* 2015, value of mean difference of testcrosses were 3.56 (x1) and 0.47 (x3) respectively. The lowest mean difference of yield of testcross progeny (Ai-Bi) in *winter* 2013 was 4.74 and in *rainy* 2015 was 0.53, as these lowest values were more than x1 (3.56) and x3 (0.47), so the heterotic pattern of all the inbreds could be identified. However, the lowest mean difference of yield of testcross progeny (Ai-Bi) in *rainy* 2014 was zero, thus greater mean difference of testcrosses during *rainy* 2014 ($x_2 = 7.52$) facilitates classification of 20 lines out of 32.

Eighteen lines showed significant SCA effect with tester CML161 (A) and were classified in heterotic group B whereas sixteen genotypes displayed significant SCA effect with tester CML165 (B) and were placed accordingly in heterotic group A. However twenty lines with significant GCA effect and the magnitude of SCA effect was almost equal with both parents, hence were assigned in AB heterotic group. Of which two lines (QIL-4-2042 and WNC 18737) recorded significant SCA effect with tester CML161 (A) and six lines (WNC 19082, QIL-4-2064-1, QIL-4-2018-1, QIL-4-2052, QIL-4-2017 and QIL-4-2248) recorded significant SCA effect with tester CML165 (B) (Table 1; online Supplementary Table S3). However, twelve inbreds (QIL-4-2032-2, QIL-4-2144, QIL-4-2065-1, QIL-4-2006, QIL-4-2022, QIL-4-2053-1, QIL-4-2066, QIL-4-2028-1, QIL-4-2034, QIL-4-2025, QIL-4-2023 and QIL-4-2050-1) could not be classified into any specific heterotic group which could be attributed to the nature of source germplasm.

### Selection of novel inbred lines based on combining ability and development of pedigree crosses

Nine of the QPM inbred lines namely QIL-4-2024, QIL-4-2184, QIL-4-2208, QIL-4-2180, QIL-4-2274, QIL-4-2248, QIL-4-2058, QIL-4-2064 and QIL-4-2164 were selected based on their GCA (12.21, 4.39, 2.60, 6.4, 0.72, 1.39, 18.15, 3.33 and 3.77, respectively; online Supplementary Table S3). Positive estimates of GCA are essential for a positive and direct contribution towards yield. These selected nine lines belonged to different heterotic groups. Three inbred lines belonged in Group A (QIL-4-2024, QIL-4-2208, QIL-4-2248), five inbred lines namely QIL-4-2058, QIL-4-2064, QIL-4-2180, QIL-4-2274 and QIL-4-2164 were


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**Table 1.** Categorization of 54 novel inbreds in the heterotic groups A, B and AB based on the GCA as well as SCA effects

| S. No. | Inbred Lines | Heterotic Group |
|---|---|---|
| 1. | QIL-4-2024, QIL-4-2050, QIL-4-2082, QIL-4-2039, QIL-4-2052, QIL-4-2023, QIL-4-2024-1, WNC 10175, QIL-4-2023, QIL-4-2026-1, QIL-4-2066-1, QIL-4-2248, QIL-4-2165, QIL-4-2208, QIL-4-2187 and QIL-4-2209 | Group- A (No. of lines:16) |
| 2. | QIL-4-2065, QIL-4-2029, QIL-4-2057, QIL-4-2026, QIL-4-2031, QIL-4-2080-1, QIL-4-2039-1, QIL-4-2077, QIL-4-2057-1, QIL-4-2216, QIL-4-2164, QIL-4-2169, DML 1302, QIL-4-2180, QIL-4-2172, QIL-4-2274, QIL-4-2064 and QIL-4-2058 | Group-B (No. of lines:18) |
| 3. | QIL-4-2047, QIL-4-2053, QIL-4-2063, QIL-4-2072, QIL-4-2085, WNC19082, QIL-4-2064-1, QIL-4-2018-1, QIL-4-2042, QIL-4-2052, QIL-4-2017, QIL-4-2038-1, WNC18737, QIL-4-2192, QIL-4-2230, QIL-4-2238, QIL-4-2211, QIL-4-2248-1, QIL-4-2261 and QIL-4-2184 | Group-AB (No. of lines:20) |

categorized in Group B and one inbred line (QIL-4-2184) belonged to AB group. Further, these selected lines were crossed within their heterotic groups. Lines within the Group A was crossed with each other to form the pedigree crosses which eventually belonged to group A. Similarly, crosses were attempted within the lines of group B and thus pedigree crosses generated belonged to group B. A wide number of crosses (6 within group A, 20 within group B, 3 in A × AB and 5 in B × AB) were generated within the heterotic groups but owing to poor germination as well as seed availability of parental lines, notably, seven pedigree crosses were developed in *rainy* season in 2017 at Ludhiana. Three pedigree crosses were developed within lines belonging to HG-A and four pedigree crosses were in HG-B as per the heterotic groups of their parental lines (online Supplementary Table S4).

### Inbred lines/progenies development from their respective crosses

Further, development of inbred lines was done for the following three years by advancing the progenies by selfing. A total of 948 families were developed with 145, 121, 152, 107, 133, 146 and 144 individual inbred lines from each of the seven pedigree crosses (online Supplementary Table S4). The 418 families from group A and 530 families from group B were developed at the initial stage. There was sequential decrease in the number of progenies developed season after season from each respective cross owing to the reason that QPM germplasm was screened in every season through light board screening method and thus the inbred lines having 25–50% opaqueness only was further advanced to next generation. There were total 948 families in the $F_2$ season, 719 in $F_3$, 705 in $F_4$, 432 in $F_5$, 147 in $F_6$, 146 in $F_7$ and 93 in $F_8$. After selfing for six seasons in three consecutive years, a total of 93 inbred lines were generated as the end product in $F_8$ generation in year 2020 in *rainy* season and thus used for their yield assessment.

### Analysis of variance for grain yield of newly derived inbred lines

The significance of mean squares for grain yield for different components of ANOVA is presented in online Supplementary Table S5 (for inbred evaluation trial). The grain yield of the inbred lines was subjected to ANOVA and it was found that treatments as well as replications were highly significant. The highly significant differences imply the presence of large genetic variation among the inbred lines. There is statistically significant difference between the means of both replications owing to the heterogeneity in land.

### Variation of grain yield and tryptophan content in inbred lines

*Per se* performance of 93 inbred lines (which were $F_8$ progenies) was assessed by conducting an inbred evaluation trial along with checks (QIL-4-2192 and HKI-163) by employing open mode of pollination in year 2020 at Ludhiana in *rainy* season. The inbred lines demonstrated a wide variability in grain yield. The grain yield of the inbred lines (q/ha) ranged from 7.11 to 32.66 q/ha. The mean grain yield was recorded as almost 26.04 q/ha. The top most performers were QIL-4-2401 (32.66 q/ha), QIL-4-2491 (31.17 q/ha), QIL-4-2469 (30.61 q/ha), QIL-4-2479 (30.06 q/ha), QIL-4-2474 (29.14 q/ha) and QIL-4-2497 (28.82 q/ha). Some of the lines did not germinate or had dead seedlings (QIL-4-2370, QIL-4-2382, QIL-4-2383, QIL-4-2387-1, QIL-4-2415, QIL-4-2508 and QIL-4-2497-1), so no grain yield was obtained in those lines. Some inbred lines were at par with the mean grain yield i.e. QIL-4-2403 (24.61 q/ha), QIL-4-2491-1 (24.95 q/ha), QIL-4-2455 (26.54 q/ha) QIL-4-2487 (26.57 q/ha) and QIL-4-2451-1 (27.0 q/ha); (online Supplementary Table S7). Additionally, biochemical analysis for tryptophan content (%) was also carried out for these inbred lines depending upon the quantity of seed required for estimation of tryptophan. Some of the inbred lines showed exceptionally high tryptophan content such as QIL-4-2439 (0.101), QIL-4-2381 (0.089), QIL-4-2379 (0.086), QIL-4-2443 (0.081) and QIL-4-2485 (0.08). Some inbred lines having low tryptophan were QIL-4-2471, QIL-4-2469, QIL-4-2386-2, QIL-4-2466, QIL-4-2473 (0.047, 0.053, 0.054, 0.054, 0.054 µg/g) content respectively (online Supplementary Table S7). So, among the set of 93 inbred lines, the best performing 22 inbred lines (Table 2) were selected based on the criteria of grain yield (q/ha) (must be more than 20 q/ha) and high tryptophan content (more than 0.6 µg/g).

### Yield performance of the heterotic crosses generated from opposite group

The 22 inbred lines (nine from Group-A and thirteen from Group-B) were selected (Table 2) and further, a total of 25 crosses were made using direct crossing (the nine inbred lines from the group A were used as females and were crossed with the thirteen inbred lines from group B which were used as males) by hand pollination in *rainy* season in 2021. Reciprocal crosses were not made, only direct crosses were done. Among the set of 25 hybrids developed, the replicated grain yield data of the 16 hybrids only (Table 3) were then evaluated (because the seed available for carrying out replicated yield trials were sufficient for these hybrids only) along with checks for grain yield in *winter* season in year 2021. The hybrid seed of the remaining nine crosses (QIL-4-2370 × QIL-4-2386, QIL-4-2380 × QIL-4-2474, QIL-4-2417 × QIL-4-2386-1, QIL-4-2487 × QIL-4-2399, QIL-4-2487 × QIL-4-2455, QIL-4-2487 × QIL-4-2457, QIL-4-2487 × QIL-4-2474, QIL-4-2487-1 × QIL-4-2399, QIL-4-2491 × QIL-4-2497) were not enough to be tested in the replicated design, however their performance was tested in the augmented design. The grain yield (q/ha) data of hybrids was subjected to ANOVA (online


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**Table 2.** Selected 22 inbred lines (among the set of 93 $F_8$ progenies) with their yield potential and tryptophan content

| S. No. | Heterotic group A (Female) | GY (q/ha) | Tryptophan Content (% Trp* per 100 g of protein) | S. No. | Heterotic group B (Male) | GY (q/ha) | Tryptophan Content (% Trp* per 100 g of protein) |
|---|---|---|---|---|---|---|---|
| 1. | QIL-4-2491 | 31.17 | 0.69 | 1. | QIL-4-2401 | 32.66 | 0.70 |
| 2. | QIL-4-2479 | 30.06 | 0.66 | 2. | QIL-4-2469 | 30.61 | 0.53 |
| 3. | QIL-4-2487-1 | 27.58 | 0.66 | 3. | QIL-4-2474 | 29.14 | 0.66 |
| 4. | QIL-4-2370 | 27.58 | 0.75 | 4. | QIL-4-2497 | 28.82 | 0.61 |
| 5. | QIL-4-2487 | 26.57 | 0.71 | 5. | QIL-4-2474-1 | 27.80 | 0.70 |
| 6. | QIL-4-2491-1 | 24.95 | 0.66 | 6. | QIL-4-2459 | 27.05 | 0.70 |
| 7. | QIL-4-2373 | 22.51 | 0.72 | 7. | QIL-4-2455 | 26.54 | 0.61 |
| 8. | QIL-4-2380 | 21.19 | 0.66 | 8. | QIL-4-2457 | 23.65 | 0.75 |
| 9. | QIL-4-2417 | 20.33 | 0.75 | 9. | QIL-4-2399 | 22.72 | 0.68 |
| | | | | 10. | QIL-4-2471 | 21.26 | 0.43 |
| | | | | 11. | QIL-4-2386-1 | 21.20 | 0.67 |
| | | | | 12. | QIL-4-2458 | 19.19 | 0.65 |
| | | | | 13. | QIL-4-2386 | 22.34 | 0.70 |

*trp:Tryptophan.

Supplementary Table S6) and it was found that treatments were highly significant, which demonstrates the presence of large genetic variation. There were non significant differences between the means of both replications. The mean grain yield of the 16 hybrids ranged between 31.4 and 81.2 q/ha (Table 3). Two standard checks, *viz*. IQPM-203 (QPM hybrid from ICAR-IIMR) and Bio-9544 (a normal maize hybrid from Bioseed Pvt. Ltd.) were also used for comparing the grain yield performance of the hybrids tested. The three cross combinations i.e. QIL-4-2380 × QIL-4-2401, QIL-4-2417 × QIL-4-2401 and QIL-4-2487-1 × QIL-4-2401 were the best performing hybrids with the grain yield of 81.2, 78.1 and 77.1 q/ha respectively as compared to the performance of checks *viz*. IQPM 203 (68.5 q/ha) and BIO 9544 (79.1 q/ha). In the present study, it is notable that QIL-4-2401 performed exceptionally good as male parent in all three above listed crosses, so it can be further exploited as a broad based tester as well as parent for development of superior hybrids which can be further commercialized or exploited based on their performance. The three best heterotic crosses identified in this study can be potentially useful in maize breeding programmes to obtain high yielding hybrids. Higher grain yield of testcrosses indicate that the inbred lines used in this study interacted positively with the genetic backgrounds of CML161 and CML165 and could be used as useful sources of favourable alleles for yield enhancement. Similarly, the testers have the potential to uncover the desirable alleles of untested germplasm for grain yield and can be utilized as potential testers in a QPM maize hybrid-breeding programme.

**Table 3.** Evaluation of grain yield performance of selected 16 hybrids developed from 9 and 13 parental lines from heterotic group-A and heterotic group-B respectively

| S. No. | Hybrid | GY (q/ha) | Ranking |
|---|---|---|---|
| 1. | QIL-4-2370 × QIL-4-2401 | 48.7 | 12 |
| 2. | QIL-4-2370 × QIL-4-2471 | 63.1 | 6 |
| 3. | QIL-4-2370 × QIL-4-2497 | 55.1 | 10 |
| 4. | QIL-4-2380 × QIL-4-2401 | 81.2 | 1 |
| 5. | QIL-4-2417 × QIL-4-2399 | 69.7 | 5 |
| 6. | QIL-4-2417 × QIL-4-2401 | 78.1 | 2 |
| 7. | QIL-4-2417 × QIL-4-2471 | 71.3 | 4 |
| 8. | QIL-4-2417 × QIL-4-2474 | 58.0 | 7 |
| 9. | QIL-4-2417 × QIL-4-2474-1 | 35.7 | 13 |
| 10. | QIL-4-2479 × QIL-4-2474 | 58.0 | 7 |
| 11. | QIL-4-2479 × QIL-4-2386 | 58.0 | 7 |
| 12. | QIL-4-2487 × QIL-4-2471 | 48.9 | 11 |
| 13. | QIL-4-2487-1 × QIL-4-2401 | 77.1 | 3 |
| 14. | QIL-4-2491 × QIL-4-2386-1 | 31.4 | 14 |
| 15. | QIL-4-2491-1 × QIL-4-2455 | 56.7 | 8 |
| 16. | QIL-4-2491-1 × QIL-4-2457 | 56.4 | 9 |
| **17.** | **IQPM-203 (Check)** | **68.5** | |
| **18.** | **Bio-9544 (Check)** | **79.1** | |

## Discussion

The majority of breeding strategies generally aim towards the development of promising and high yielding QPM cultivars. The availability of efficient testers which can classify the inbred lines into different heterotic groups is an effective way to develop high-yielding hybrids and synthetic varieties (Annor *et al*., 2019). Our research stands out differently at many points when it is compared to earlier literature cited. First and foremost, in majority of the past studies on heterotic grouping of germplasm, the work plan consisted of process involving selection and crossing of inbred lines with testers followed by their heterotic grouping which usually takes one or two years to complete. Our research was done over a long time span of almost nine years


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(2013–2021) which was initiated with the development of test crosses (*rainy* 2013, *winter* 2013 and *winter* 2014) and their evaluation in *winter* 2013, *rainy* 2014 and 2015 using two testers (CML 161 and CML 165) and 66 inbred lines followed by the classification of inbred lines into distinct heterotic groups. The mean grain yield of testcrosses varied on a large scale in all three seasons and the inbred lines having superior yield of more than 100 q/ha was thus identified. Based on the combining ability, the inbred lines were classified into A, B and AB groups. Further, best inbred lines were identified on the basis of GCA and pedigree crosses were developed in rainy season in 2017. The pedigree crosses based inbred line generation was done for the next three years (2017–2020). As much as total 948 inbred lines were developed in the $F_1$ generation. Raising of such large generations season after season from several crosses is a laborious task as well as a critical aspect also which involves multiple resources.

After *per se* yield assessment of 93 ($F_8$ progenies) lines in rainy season in 2020, 22 best inbred lines were selected based on the grain yield and tryptophan content. 25 heterotic crosses were generated in rainy season in 2021 by crossing lines with their opposite heterotic group. Yield performance of the hybrids was assessed in winter season in 2021 and three QPM hybrids (QIL-4-2380 × QIL-4-2401, QIL-4-2417 × QIL-4-2401 and QIL-4-2487-1 × QIL-4-2401) were found to be the best performing hybrids. QIL-4-2401 was identified as exceptionally good as male parent in all three above listed crosses. The results of the current study revealed that as most of the inbreds (among the 66 lines) were derived from available superior hybrids so that probably resulted in heterotic genotypes with mixed alleles from opposite heterotic groups.

Furthermore, this study focuses both on GCA as well as SCA, judged as the necessary pillars for heterotic grouping. In the panel of 66 lines, they were heterotic grouped based on both GCA and SCA. The best nine lines among these were selected on basis of positive GCA. Most of the researchers initiated their work using the less number of lines followed by their crossing with testers. In present study, a set of 66 lines was used as the base panel for crossing with two testers for the further studies. Moreover, in the literature cited, not much detailed work has been done on the QPM in light of the heterotic grouping. We have also conducted the analysis of quality parameters (tryptophan content) of maize inbred lines. As *o2* mutant has pleiotropic effects which results in low grain yield, larger germ size and reduction in grain weight (Babu and Prasanna, 2013), so it is difficult to achieve the desired levels of both high yield as well as optimum tryptophan content. We have identified 22 inbred lines with grain yield (q/ha) (more than 20 q/ha) and high tryptophan content (more than 0.6 µg/g). QPM hybrids were developed by using these inbreds as the parental lines and then assessed for grain yield. QPM promises the nutritional security as well as high consumer acceptance, so identified hybrids can be further tested in the variety release pipeline and thus can be promoted for commercial release.

There were many studies carried out in the past which categorized the breeding material into different groups. The combining ability plays an important role in selecting superior parents for hybrid combinations and in studying the nature of genetic variation present (Koutsika-Sotiriou, 1999). Pixley and Bjarnason (1993) evaluated QPM inbred lines across environments and reported significant GCA effects for grain yield. Similarly, Vasal (1994) evaluated 10 QPM parents in a diallel study and GCA effects were highly significant for grain yield and other studied traits. Vasal (2000) carried out line × tester analysis using 92 test crosses generated by crossing 23 partially inbred lines with four testers to estimate the combining ability of lines and identified 12 lines with positive GCA effects across environments and significant SCA effect for grain yield. Iqbal *et al.* (2007) and Shams *et al.* (2010) also reported significant SCA effects in most of the crosses for grain yield in maize. Barata and Carena (2006) conducted a similar study as Menkir *et al.* (2004) to classify 13 elite North Dakota maize inbred lines into current U.S. Corn Belt heterotic groups. In a study by Bidhendi *et al.* (2012), using both the Griffing method and the biplot approach for diallel analysis, the lines derived from LSC (Lancaster sure crop) was identified as the best lines based on significant positive GCA effects, The maximum best-parent heterosis values and highest SCA effects resulted from crosses B73 × MO17 and A679 × MO17 for GY.

In line with the present study, in a study conducted by Nepir *et al.* (2015), inbred lines L12, L17, L19, and L20 had desirable GCA effects for grain yield, whereas on the other hand, for protein quality traits, the inbred lines L12 and L13 were identified as the best general combiners. Hybrids L17 × 142-1eQ and L20 × 142-1-eQ were found to have the most desirable *per se* performances as well as SCA effects for grain yield. In a study by Fan *et al.* (2016), the three testers i.e. TRL60 tester, YML146 and TR2 were used to generate 12, 8 and 7 test crosses. All eight test-crosses from YML146 tester (a line from Suwan1 heterotic group) had 10% higher grain yield than that of the check. Through the literature, Line YML146 was identified by Fan *et al.* (2008) while conducting a study of 100 crosses using 25 temperate maize germplasm and four germplasm accessions from CIMMYT. It was noticed that YML146 was a good male line that can be used directly in hybrid development (Fan *et al.*, 2009, 2014, 2015).

GCA can be more informative and helpful in getting more superior cross combinations. The selected cross combinations in this study can be tested in multi-location trials for assessing their performance across diverse ecologies. Based on the grain yield performance, these identified hybrids can be further advanced towards development of hybrids on commercial scale. Also, better performing crosses along with inbred lines having high GCA were successfully identified in this study, this can serve as potential reservoir of the germplasm which can further be used in more research activities. The parental lines of the hybrids can also be used in other breeding programmes which aim towards achieving other breeding goals. Moreover, heterotic grouping is also categorized as the way for exploring the genetic potential of inbred lines as well as maximizing genetic gains. Crosses with highly positive and significant estimates of SCA with superior yield could be tested for stability performance in multi-location trials. Though significant SCA effect was revealed by these lines, presence of significant GCA effect indicate the inability of used testers to discriminate their heterotic pattern and lines combined well with both the testers. Though lines with significant GCA effect shared both the heterotic groups, lines with both significant and positive GCA and SCA effects could serve as parents in hybrid programmes due to their potential as good combiner.

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