International Journal of Agriculture and Environmental Research

ISSN: 2455-6939

Volume:03, Issue:05 "September-October 2017"

EFFECT OF RECURRENT BACKGROUND ON THE PROTEIN QUALITY OF QPM LINES DEVELOPED THROUGH MARKER ASSISTED BACKCROSS BREEDING (MABB)

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ABSTRACT

Quality Protein Maize (QPM) is the modified version of maize lines introgressed with *opaque-2* gene along with numerous modifiers for kernel hardness to quench its earlier pleiotropic effects, including low nutritive value of endosperm protein. In the present investigation we reported the effect of recurrent parent genome on the quality of protein in QPM lines developed through marker assisted backcross breeding (MABB). Two generation MABB program for incorporation of the *opaque-2* gene along with phenotypic selection for kernel modification in the background of normal maize inbred lines Pant11R 126, Pant 11R 128 and Pant 11R 53 using CIMMYT lines as donor parents namely CML 162 and CML 189 resulted in development of paired crosses among polymorphic lines at *opaque-2* locus. BC_2F_2 seeds with >25 % opaqueness were selfed and the protein and tryptophan concentration in endosperm protein of BC_2F_3 lines was reported to lie in the range of 8.23–9.16 % and 0.075-0.096 % respectively. As evident from the results there was a significant effect of background found on the converted QPM lines.

Keywords: Quality Protein Maize, Marker assisted backcross breeding, *opaque-2*

INTRODUCTION

Maize is an important food and animal feed in many parts of the world, but the deficiency in essential amino acids, particularly lysine and tryptophan, limit its nutritional value. The significant breakthrough came in 1964 when Mertz and his coworkers at Purdue University, USA, established the nutritional superiority of *opaque-2 (o2)* mutant with an enhanced concentration of lysine and tryptophan **(Mertz *et al.* 1964).** This finding provided an opportunity

ISSN: 2455-6939

Volume:03, Issue:05 "September-October 2017"

for breeding new cultivars with high lysine protein **(Bjarnason and Vasal 1992; Prasanna *et al.* 2001).** The *opaque-2* grain was chalky, not shiny, ears were small, its yield were 8-15% lower than the traditional maize varieties, more susceptible to fungi and insects, both in fields and in storage and is dried more slowly. It weighed less than the normal maize kernel due to air spaces surrounded its loosely packed starch granules. Due to these reasons *opaque-2* maize lost its appeal. Hardly anybody seemed to like it. Farmers refused to grow it because of its poor field performance—even millers resisted handling it because of poor storage characteristics. By late 1970's the *opaque-2* maize had been discredited. CIMMYT breeders successfully combined the high lysine potential of *opaque-2* with genetic endosperm modifiers, releasing new maize genotypes which are collectively referred to as Quality Protein Maize (QPM) **(Vasal *et al.* 1993; Prasanna *et al.* 2001)**. In 2000, the World Food Prize was awarded to Villegas and Vasal for their efforts to develop QPM for worldwide use **(Vietmeyer, 2000).** India was first among the countries in the world to focus on improvement of maize quality soon after the nutritional benefits of *opaque-2* mutation was brought to light. As a result of a research programme initiated in 1966 under the AICMIP, three *opaque-2* composites, namely Shakti, Rattan and Protina were developed and commercially released in 1970. Since 1998, intensive efforts have begun at various centers in the country under the NATP resulting in release of two QPM hybrids, Shaktiman-3 and Shaktiman-4, Shaktiman-5 and HQPM-1 with the CIMMYT QPM inbreds as parental lines. Recent investigations into the improved protein quality of the *opaque-2* mutant and the genetic mechanisms that can suppress its starchy kernel phenotype provide new insights to support the continued improvement of QPM **(Gibbon, 2005).** QPM development process could be greatly accelerated if the identities of *o2* modifier genes were known, but unfortunately relatively little is known about their number, chromosomal location, and mode of action. Many of these genes had a complex system of genetic control and spread throughout all the ten chromosomes with dosage effects and cytoplasmic effect **(Belousov 1987).** Multigenic effects have been reported controlling amino acid content and genes governing the levels of lysine have been mapped on chromosome 2, 4 and 7 **(Wang *et al.*, 2001 and Wu *et al.*, 2002).** Such genes have been identified and include eEF1A (7L), eEF1A (4S), eEF1A (2S), FAA (1L), FAA (2S), FAA (2L), FAA (3S), FAA (4L), FAA (5L), FAA (7L), FAA (8S) and FAA (9S). Holding *et al.* 2008 found seven quantitative trait loci (QTLs) based on simple interval mapping and showed that chromosomes 7 and 9 form the major hub of *o2* modifiers. Yang *et al.* 2005 reported QTLs linked to opaque-16 (*o16*) gene influencing the lysine content were on chromosome 8 in the $F_{2:3}$ populations. Gibbon *et al.* 2003 showed that vitreousness and amino acid composition of the maize endosperm may be positively correlated and they also found seven QTLs on chromosomes 1, 5, 7, 9 and 10. Holding *et al.* 2011 characterized *o2* modifier QTLs and candidate genes in the recombinant inbred lines for density, vitreousness and breakage mean, mapped to chromosome 5, 7 and 10. The recombinant inbred lines (RIL) population using QPM x QPM inbred are very

ISSN: 2455-6939

Volume:03, Issue:05 "September-October 2017"

useful in high-resolution mapping for *o2* modifiers influencing the tryptophan content and based on simple interval mapping using QTL cartographer, six QTLs associated with tryptophan content were recently identified on chromosomes 5, 7 and 9 **(Babu *et al.*, 2015).** Two QPM inbreds, derived from the same parent (CM145), significantly differed in tryptophan content to a large extent (VQL2, 0.54; VQL8, 0.94), which may be due to the influence of *o2* modifiers **(Babu *et al.*, 2005).** The effect of background thus plays an important role in the tryptophan and lysine content as evidenced from the discussion done above. Hence our present investigation was to study the effect of different recurrent parents on the tryptophan content of BC_2F_3 lines derived from them using common QPM donor line from CIMMYT.

MATERIALS AND METHODS

Normal maize inbred lines Pant 11R-53, Pant 11R-126 and Pant 11R-128 were used as recipient parent (recurrent) while CML189 and CML 162 developed by CIMMYT were used as QPM donor parent for *opaque-2* allele. The above said inbred lines were analyzed for polymorphism with *opaque-2* gene specific SSR markers at the *opaque-2* locus. These markers were *phi057, phi112,* and *umc1066* (www.maizegdb.org). DNA isolation was carried out using the CTAB method **(Doyle and Doyle, 1990)** with some modifications. PCR amplification was performed using Thermal cycler (Applied Bio systems, U.S.A) and cycling consisted of initial denaturation at 94^0C for 2 min, followed by 35 cycles of amplification at 94^o C for 40 sec, 55–60^0 C for 40 sec, and 72^0 C for 1 min. A final extension step at 72^0 C for 7 min was followed by termination of the cycle at 4^0C. The amplified products (15 µl) were resolved on a 3.5% Metaphor gel at 110 V for 3-4 hours according to **Senior *et al*. (1998)** using GeNei Maxi electrophoretic unit. The gels were photographed using PC based gel documentation system ALPHA IMAGER.

Development of Back Cross populations

Breeding programme involved development of F_1 seeds from the paired crosses with Pant 11R-53, Pant 11R-126, Pant 11R-128 used as recipient of *opaque-2* allele and CML189 and CML 162 as donors. At the time of maturity, ears on normal maize line consisted of crossed seed (F_1 of normal x CML lines) were harvested. Normal maize lines were used as recurrent parent and CML189 and CML 162 as pollen source to generate BC_1F_1 seeds. Each BC_1F_1 plants were screened using SSR marker *phi057* before flowering and selected heterozygous plants at *opaque-2* locus were sown in ear to row fashion to develop BC_2F_1 population. Before flowering, genomic DNA from individual plants was isolated and genotyped using SSR marker *phi057* and plants with *opaque-2* allele in heterozygous condition were selfed to develop BC_2F_2 seeds. Further, these plants were screened phenotypically and agronomically superior plants similar to recurrent parents were retained only. The BC_2F_2 seed harvested on BC_2F_1 plants were screened after

ISSN: 2455-6939

Volume:03, Issue:05 "September-October 2017"

drying well using white light box table which helps in categorization of kernels with different gradient of opaqueness. Kernels from each ear separately were thus exposed with fluorescent light and categorized into kernels without opaqueness, kernels with 25%, 50%, 75% and 100% opaqueness. Kernels with >25% opaqueness were selected and planted in ear to row and selfed to generate F_2 derived BC_2F_3 seeds.

Protein Estimation

Nearly about 20-50 mg of the sample (very fine powder of kernel endosperm i.e. without embryo) was placed on the nitrogen free foil and folded in a tabulated form. Each tablet was placed in the Rapid N-cube Analyzer and keeping urea as standard the free nitrogen was estimated by the instrument. The ability to analyze large samples was essential for the use of the Dumas method for routine N or protein determination of maize samples which is based on the principle of quantitative combustion digestion of the sample at approx. 960°C in excess O_2. The bound nitrogen is transferred into molecular nitrogen and nitric oxides. The analysis gases are transferred with CO_2 as carrier gas via a catalytic post combustion zone onto a reduction zone. At this point, the conversion of the nitric oxides into nitrogen at hot tungsten takes place. Furthermore, the excess oxygen is bound. After a two stage drying, the gas mixture flows to the thermo conductivity detector (TCD) via an electronic flow controller. A connected PC calculates the N concentration in the sample from the TCD signal of the N_2 in the CO_2 and from the sample weight. (Protein (%) = Nitrogen (%) x 6.25)

Tryptophan Estimation

Grinded 30 seeds of each sample to a very fine powder using grinding mill. The sieved (0.5 mm) fine flour of each genotype was packed in water proof seed packets and stored at 4 °C. The milled flour was then defatted with hexane in a Soxhlet-type continuous extractor for 6 h. After hexane evaporation, 80 mg of powder was digested using 3 mL of 4 mg/mL papain. A blank with only papain solution was included as a control. The tubes were incubated at 65 ^oC for 16 h (shaken at least twice in the first hour of incubation), allowed to cool to room temperature, and centrifuged at 3600g for 10 min, ensuring a very clear supernatant. One milliliter of the hydrolysate (supernatant) was carefully transferred to a clean tube, and 3 mL of reagent D (colorimetric reagent) was added as described by the standard protocol of **Nurit *et al.*, 2009**. Samples were thoroughly stirred (vortexed) and then incubated for 30 min at 65 ^oC. The samples were allowed to cool to room temperature before reading their optical density (OD) at 560 nm in a UV-Visual Double Beam Spectrophotometer. Each sample was analyzed in triplicate to ensure accuracy.

ISSN: 2455-6939

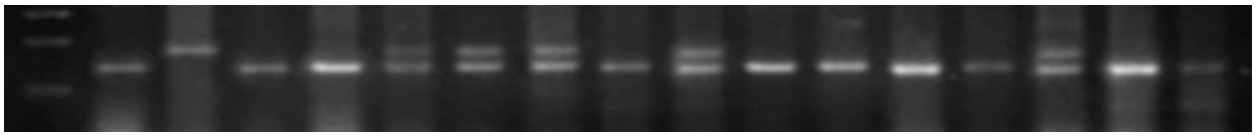
Volume:03, Issue:05 "September-October 2017"

RESULTS AND DISCUSSION

SSR markers namely *phi112*, *phi057* and *umc1066* have been reported for detection of polymorphism as well as monitoring of inheritance of *o2* gene. In our present investigation we found that the SSR marker, *phi112* exhibited dominant polymorphism between the normal and high lysine and tryptophan maize inbred lines. The parental lines Pant 11R-128, Pant 11R-126 and Pant 11R-53 had 150bp, 160bp and 165 bp respectively *phi057* marker allele size at *o2* locus while the donor lines CML 162 and CML 189 had approximately 170bp as indicated in Fig 1. Thus, the marker *phi112* clearly exhibited dominant inheritance and cannot be used in conversion programme since the marker is unable to distinguish plants heterozygote for *o2* locus from other plants homozygous for *o2* locus in backcross population. The SSR marker *phi057* exhibited stable and discrete phenotype in the present investigation and therefore considered to be more reliable whereas, variable and ambiguous phenotype with *umc1066* indicated that this marker cannot be used reliably in selection of plants during the conversion programme.

Fig 1: Polymorphism at *o2* locus using *Phi057* SSR marker within Pant inbred recurrent parents and CML donors.

Fig 2: BC_1F_1 population screening of cross between parents using SSR marker *Phi057*. M-Molecular weight marker, P1-Pant 11R 128; P2-CML 189; BC_1F_1: Backcrossed Progenies of F_1



Marker assisted background analysis of the BC/recombinant progenies is useful in determining the relative contribution of donor parent. In the present study 30 SSR markers were screened to identify polymorphic markers to be used in recovery of the recurrent parent genome. Of these, 17 markers were found to be polymorphic and used for the background selection along with the phenotypic observation for recurrent phenotype. When Pant 11R-128 was one parent with CML162 and CML189 as other parents involved in crossing 38 and 43 plants were scored to be heterozygous. In the crosses where Pant 11R-126 was used as seed parent with CML162 and CML 189 as pollen parent, the number of heterozygous plants at *o2* locus in BC1F1 population observed to be 48 and 49, respectively. The BC_1F_1 population of cross between Pant 11R-53 x

CML162 and Pant 11R-53 x CML189 had 47 and 37 plants heterozygous at *o2* locus. In BC_2F_1 populations where Pant 11R-128 was used as seed parent in combination with QPM donor lines CML162 and CML189, the number of plants exhibited heterozygous genotype at *o2* locus were 46 and 43, respectively. Two BC_2F_1 populations where Pant 11R-53 was a common parent in crosses with CML162 and CML 189 exhibited 48 and 37 plants heterozygous at *o2* locus, respectively. The BC_2F_1 progenies of a cross between Pant 11R-126 and CML162 and Pant 11R-126 and CML189 had 47 and 51 plants heterozygous at *o2* locus, respectively. Selfing of the BC_2F_1 populations of the above said crosses resulted in BC_2F_2 seeds which were again selected for > 25% opaqueness under light box to derive the BC_2F_3 seeds, used for the protein and tryptophan analysis.

The average per cent protein in the BC_2F_3 lines derived from the crosses between Pant 11R-53 and CML 189 was 8.766 whereas lines derived from Pant11R-126xCML189 had mean protein content of 8.738 %. It was observed that when Pant 11R-128 was used as the recipient parent with donor CML189 the mean protein per cent in BC_2F_3 line was 8.505. Similarly, the BC_2F_3 lines derived from the cross between Pant11R-126 and CML162 had average protein content of 8.806 %. Mean protein content in Pant11R-128xCML162 derived BC_2F_3 line was determined to be 8.875 %. Besides it when CML 162 was used as a donor for *o2* gene, Pant11R-53 xCML162-BC_2F_3.population had a mean protein per cent of 9.073 which was the maximum among all crosses derived from it. The supposition of well proven and well established relationship between the tryptophan and lysine that they are highly correlated and as such an assay for either amino acid can be used for analyzing protein quality, although in practice the latter is most often chosen due to lower laboratory costs **(Hernandez and Bates 1969, Villegas *et al.*, 1992, Nurit *et al.*, 2009).** Lysine and tryptophan concentration in maize kernels of agronomically advanced QPM lines are highly correlated. A 4:1 ratio of lysine to tryptophan has been reported in normal and quality protein maize **(Bjarnason and Vasal 1992; Vivek *et al.*, 2008; Cordova 2001).**

The maximum tryptophan content derived from cross between Pant 11R-128 and CML189 was 0.075% with an average mean tryptophan of 0.064 % in (Pant 11R-128x CML189) BC_2F_3 population. Pant11R-126xCML189-BC_2F_3:3 with 0.079% tryptophan was identified to be QPM line derived from the cross between Pant11R-126 and CML189. In case where Pant11R-53 and CML 189 were used to generate population, recombinant line Pant11R-53xCML189-BC_2F_3:3 (0.096 %) identified to possess similar to QPM yardstick. The average tryptophan content in lines derived from Pant 11R-126x CML189 and Pant 11R-53x CML189 crosses were 0.069% and 0.077 % respectively. In population where CML 162 was used as QPM donor and Pant11R-53 as recurrent parent the line, Pant11R-53xCML162-BC_2F_3:1 (0.091%) was identified to be QPM type since it had tryptophan higher that 0.075%. The line namely Pant11R-126xCML162-BC_2F_3:5 (0.083%) derived from cross between Pant11R-126 and CML162 exhibited tryptophan

ISSN: 2455-6939

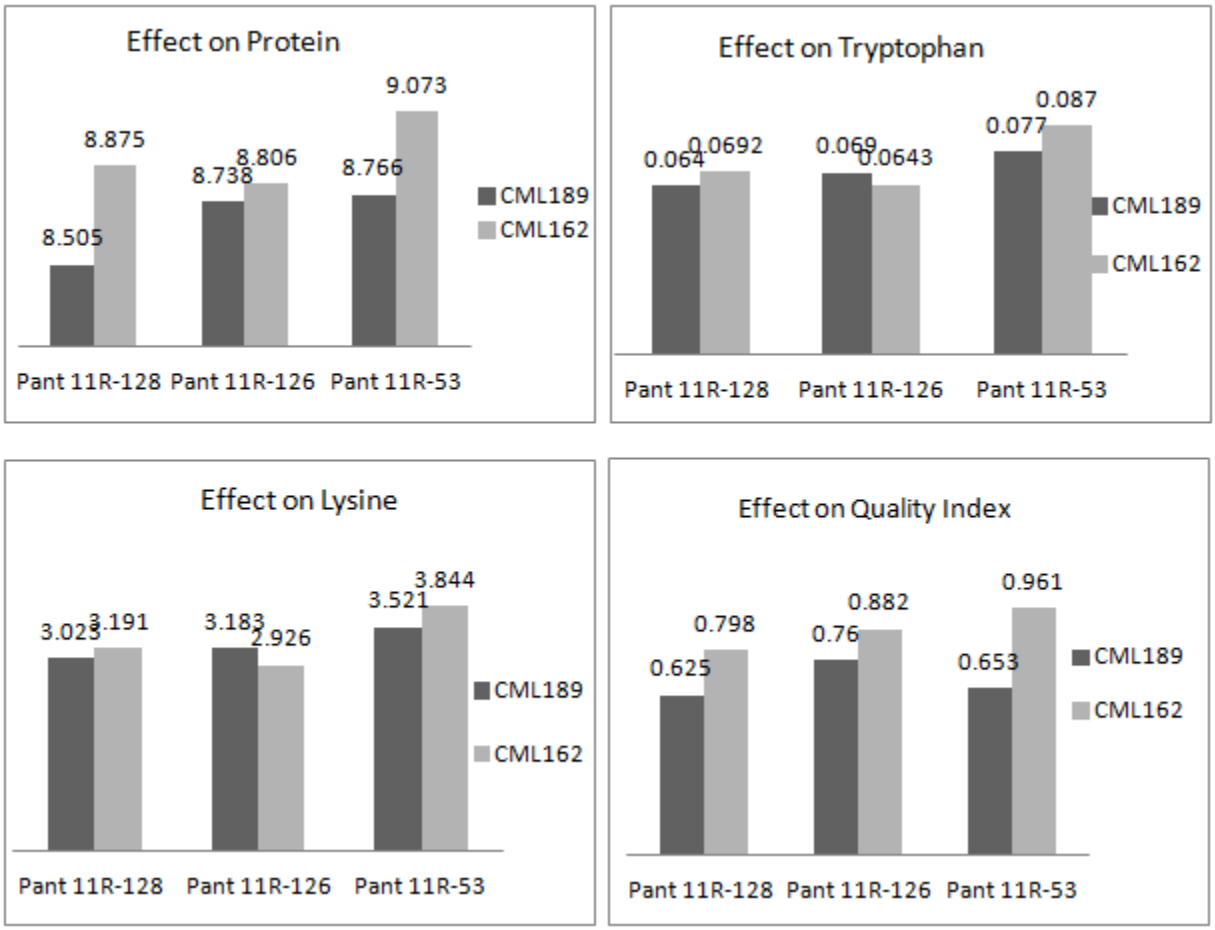
Volume:03, Issue:05 "September-October 2017"

content higher than 0.075 whereas line namely Pant11R-128xCML162-BC_2F_3:1 (0.084%) derived from cross between Pant11R-128 and CML 162 possessed higher tryptophan and identified to be QPM line. Quality index (QI) is one of the main criteria used to determine whether a line is QPM or not. The QI is determined considering both protein and tryptophan of a sample as indicated in Fig. 3 and Fig. 4. In view of the growing importance of QPM in India and to hasten the pace of progress of QPM cultivar development, it is important to develop more heterotic combinations with improved nutritional quality and kernel texture. In this direction, conversion of nonQPM inbred lines into QPM versions is an important viable option due to its quicker development, similar and tested heterotic potential, combining ability and adaptability to existing nonQPM hybrids. Utilization of QPM could substitute protein additives which are used in animal feed composites, reducing its cost **(Scott *et al.*, 2009).** Besides it, the rapid advances in genome research and molecular technology have led to the use of molecular marker- assisted selection (MAS) in enhancing selection efficiency and expediting the development of new cultivars with higher yield potential **(Ribaut and Hoisington 1998: Babu *et al.* 2005).** Moreover, with the recent development of PCR- based markers, for example, simple sequence repeats (SSRs) and single nucleotide polymorphism (SNPs) a substantial improvement in the capacity to efficiently screen large populations has been achieved, thereby increasing the efficiency of the MAS experiments. Similarly the studies have been carried out where the lysine levels in normal and QPM maize averaged 2.0 % and 4.0 % of total protein in whole grain flour respectively, but range across genetic backgrounds from 1.6 - 2.6 % in normal maize and 2.7 - 4.5 % in their *o2* converted counterparts **(Moro *et al.*, 1996).** In another investigation six newly developed QPM lines were assessed and per cent crude protein varied between 6.22 and 8.75 % while % lysine and tryptophan were in the range of 3.31-3.72 and 0.49 to 0.87 respectively in different genetic backgrounds **(Olakojo *et al.*, 2007).** Thereafter, in the present investigation we chose to determine the background effect of recurrent parent inbred lines when same donor parent is used for deriving the BC_2F_3 populations. This was done at the biochemical level by determining the protein and tryptophan content in the derived lines of crosses among Pant 11R-126, Pant 11R-128 and Pant 11R-53 with CML162 and CML 189 as donors respectively. The present investigation has been successfully integrated molecular marker and phenotypic assisted selection approaches in conversion of normal maize in QPM lines. The lines identified to be promising have potential as germplasm in development of single cross QPM hybrids.

ISSN: 2455-6939

Volume:03, Issue:05 "September-October 2017"

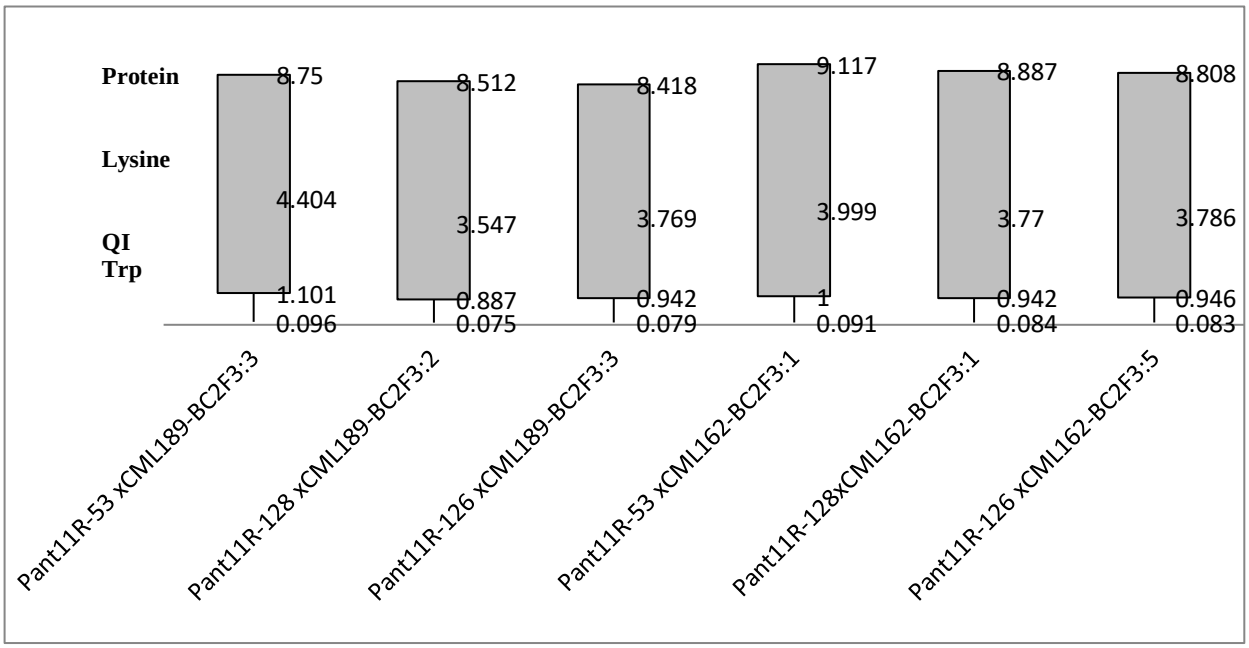
Fig 3: Background effect on BC_2F_3 derived lines



ISSN: 2455-6939

Volume:03, Issue:05 "September-October 2017"

Fig 4: QPM lines with maximum protein and tryptophan content in BC_2F_3 lines



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Volume:03, Issue:05 "September-October 2017"

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