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Development of low seed β-ODAP containing *Lathyrus sativus* genotypes with higher seed yield

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Abstract
Lathyrus sativus is nutritive hardy pulse crop. The major constraints of *Lathyrus sativus* is the presence of neuro-toxic non-proteinogenic amino acid β-N-oxalyl-L-α, β-diaminopropionic acid (β-ODAP) and low yield. Hence, it is inevitable to select the low β-ODAP content *Lathyrus sativus* for safe consumption. Therefore, after screening diverse genotypes, a breeding population as developed by crossing low ODAP containing Maharera and high ODAP containing RLK-1950 to identify low seed β-ODAP containing genotypes with higher seed yield. Phenotypic traits and seed β-ODAP content of the segregating population (F3) were evaluated. It was found that biological yield per plant was significantly and positively correlated to pod yield per plant, seed yield per plant and seed β-ODAP content. Similarly, pod yield per plant was significantly and positively correlated to biological yield per plant, seed yield per plant and seed β-ODAP content. However, seed yield per plant and seed β-ODAP content had positive but non - significant correlation. Considering higher seed yield per plant and low seed β-ODAP content (<0.3%), four Recombinant Inbreed (RI) F3 lines *viz*. RIL no. 29, 120, 134 and 189 were identified. These lines will be used for further generation advancement and yield evaluation.

Keywords: *Lathyrus sativus*, seed yield, seed β-odap content, pod yield

1. Introduction
Lathyrus sativus is a nutritive pulse crop, which requires minimum agronomic inputs and has ability to give good yield under adverse climatic condition. It can be used for human consumption as well as animal fodder. Hence, it is regarded as an integral component of subsistence agriculture in countries such as India, Bangladesh, Nepal and Ethiopia (Shiv Kumar *et al.*, 2011) [14]. In India, it is cultivated mainly in Bihar, Madhya Pradesh, Maharashtra, West Bengal, and Chhattisgarh (Khandare *et al*., 2014) [11]. Furthermore, *Lathyrus sativus* has enormous potential as a functional food to improve health conditions associated with cardiovascular disease, hypoxia as well as hypertension (Khandare *et al*., 2014 and Singh *et al*., 2013) [11, 20]. It can also fix atmospheric nitrogen through symbiotic association with rhizobia, thereby maintaining the soil nitrogen balance. In addition to this, *Lathyrus sativus* is well-known for high protein content and its seed is highly nutritious, consisting of 28% protein, 48% starch and less than 1% fat. (Xu *et al*., 2017) [22]. However, prolonged consumption of *Lathyrus sativus* has been known to cause lathyrism, a neurodegenerative syndrome due to presence of neuro-toxic nonproteinogenic amino acid β - N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) (Rao *et al.*, 1964) [18]. Therefore, its cultivation has been banned in some of the countries despite of its ability to survive under harsh climatic condition. The area of cultivation, production and productivity during 2014-15 were 305.22 ha, 297.91 MT and 976 Kg/ha respectively, however, its area of cultivation, production and productivity were reported as 273.77 ha, 172.48 MT and 630 kg/ha respectively during 2015-16, indicating decrease in area, production and productivity (agriportal.cg.nic.in). *Lathyrus sativus* being a nutritious crop and hardy to grow in low-input situation, have been preferred by small farmers. However, the association with the neurotoxic β-ODAP has led to fewer efforts on genetic improvement of *Lathyrus sativus*. Dahiya *et al*., 1976 [8] also indicated that low ODAP content lines had low yield while high yielding had high ODAP content. Although the β-ODAP and yield is known to be correlated but variability exists in the *Lathyrus* germplasm. The genetic variation can be exploited to identify *Lathyrus sativus* genotypes with low seed β-ODAP content and higher seed yield. Hence an experiment was conducted to select the high yielding low ODAP content *Lathyrus sativus* genotypes.

The segregating population (F3), derived from Maharera X RLK-1950 was subjected to field evaluation as well as estimation of seed β-ODAP. The correlation among biological yield per plant, pod yield per plant, seed yield and seed ODAP content was also analyzed.

2. Material and Methods

Field experiment were carried out in the research field of the Department of Plant Molecular Biology and Biotechnology, College of Agriculture, under the Directorate of Research Service (DSR), Indira Gandhi Krishi Vishwavidyalaya, Raipur in rabi season during 2018-19 to record biological weight per plant, pod weight per plant, seed weight per plant as well as to estimate seed β- ODAP content of 275 *Lathyrus sativus* genotypes of segregating (F3) population derived from Maharera X RLK-1950, where Maharera is known to have low β-ODAP content while RLK-1950 has higher β-ODAP content.

In this study, 275 *Lathyrus sativus* genotypes were grown in the field in rows, with each row consisting each F3 line during 2018-19. The phenotypic traits *viz*. biological yield per plant, pod yield per plant, seed yield per plant and seed pigmentation were recorded at maturity. Alongside, the low β-ODAP content Maharera and high β-ODAP content RLK-1950 were also grown in the field and above mentioned traits of these two varieties were recorded. The field image of Maharera X RLK-1950 (F3) segregating population is presented in the Figure1.

Seed β-ODAP estimation of 275 genotypes was carried out, using modified protocol of (Rao *et al*., 1978 and Aletor *et al*., 1994) [19, 4]. Chemicals *viz*., 3N potassium hydroxide (KOH), 0.5 M potassium tetraborate (K2B4O7), O- phathaldehyde (OPT), 60% ethanol, absolute ethanol and β-mercaptoethanol were used to carry out the procedure of β-ODAP estimation. Meanwhile, DL-2, 3-diaminopropionic acid monohydrochloride (L-DAP) was used to construct the standard curve. In order to determine β-ODAP content, spectrophotometer absorbance readings of each F3 line sample solution along with three types of blanks *viz*. sample blank, OPT blank and buffer blank were recorded at 425 nm, using Systronics spectrophotometer. OPT reagent is required for ODAP estimation and it should be freshly prepared. For preparation of OPT reagent, 100 mg of OPT (O-phathaldehyde) was dissolved in 90 ml of K2B4O7 along with 1 ml 99% ethanol and 0.2 ml (200 µl) of β-mercaptoethanol, then its volume was made up to 100 ml by potassium tetraborate (K2B4O7) with pH 9.9. 1% L-DAP stock solution was prepared by dissolving 300mg of L-DAP in 30 ml of solution (consisting of 20 ml 3N KOH and 10 ml 60% Ethanol) and 1% L-DAP stock solution was used for the construction of standard curve.

To construct the standard curve, 6 ml solution (consisting of 4 ml of 3N KOH and 2 ml of 60% ethanol) was poured in each of six falcon tubes. Then, 6 µl, 12 µl, 18µl, 24 µl, 30 µl and 36 µl of 1% L-DAP solution were added to 1st, 2nd, 3rd, 4th, 5th and 6th falcon tube consisting 6ml solution, making the concentration of solution 0.01 mg/ml, 0.02 mg/ml, 0.03 mg/ml, 0.04 mg/ml, 0.05 mg/ml and 0.06 mg/ml respectively. After incubating these solutions in water bath at 95 ºC for 30 minutes, observation of absorbance reading at 425nm were recorded. The standard curve with (R2=0.992) was constructed. Sample blank was prepared by mixing 250 µL of non-hydrolyzed solution (60% ethanol), 2 ml $K_2B_4O_7$ and 750 µL double distilled water. Then, buffer blank was prepared by mixing 250 µL hydrolyzing solution (2:1 of KOH and 60% ethanol solution) 2 ml $K_2B_4O_7$ and 750 µL double distilled water. The OPT blank was prepared by mixing 250 µL of non-hydrolyzed solution (60% ethanol), 2 ml OPT reagent and 750 µL double distilled water. For seed ODAP estimation, 500 mg of finely grounded seed samples of each F3 line was dissolved in 10 ml 60% ethanol by vigorous mixing. After keeping overnight, 2 ml supernatant from solution of each genotype was transferred to 2 ml eppendorf tubes, followed by centrifugation at 4500 rpm at 25 °C for 5 minutes. After transferring the supernatant to 15 ml falcon tubes, it was hydrolyzed with 4 ml of 3N KOH and was sustained in the water bath at 95 ºC for 30 minutes. 250 µL of hydrolyzed sample solutions (after cooling down), 2 ml OPT solution as well as 750 µL double distilled water were dispensed in the borosilicate bottles. Then, all the sample solutions along with three blank solutions *viz*. sample blank, OPT blank and buffer blank were incubated for 2 hours at 37-38 ºC. Then the absorbance readings of all the sample solutions along with blank solutions at 425 nm wavelength in the systronics spectrophotometer. Further, the final absorbance readings of sample solutions were calculated, using the formula,

Final absorbance = (Sample Solution-Buffer blank) – 1/3(OPT Blank – Sample Blank).

Finally, β-ODAP content of all the 275 samples in terms of percentage (%) were calculated by using final absorbance readings, linear equation of standard curve, multiplied by correction factor 1.69 and further multiplied by 100. (Aletor *et al*., 1994) [4].

Fig 1: Segregating (F3) population, Maharera X RLK-1950 (During 2018-19).

3. Results and Discussion

3.1 Phenotypic traits and estimation of seed β-ODAP content

In this study, we evaluated the segregating population (F3) of *Lathyrus sativus*, derived from Maharera X RLK-1950, consisting of 275 genotypes. Each of the F3 line was grown in the field in rows, each row consisting of each F3 line, alongside with Maharera (low β-ODAP) and RLK-1950 (high β-ODAP). After harvesting, the traits such as such as biological yield per plant, pod yield per plant, seed yield per plant and seed pigmentation of each F3 line were recorded.

And above mentioned phenotypic traits of the two parents, Maharera and RLK-1950 were also recorded. The mean biological yield per plant of segregating (F3) population was found to be 12.01 g while the biological yield per plant of Maharera and RLK-1950 were 17.20g and 20.2 g respectively. The biological yield per plant ranged between 3.84 g and 20.78 g respectively. The mean pod yield per plant of segregating (F3) population was found to be 5.39 g while the pod yield per plant of Maharera and RLK-1950 were 7.93 g and 8.61 g respectively. The pod yield per plant ranged between 1.91 g and 11.08 g. Further, the mean seed yield per plant of segregating (F3) population was found to be 4.39 g while the seed yield per plant of Maharera and RLK-1950 were 5.64 g and 3.33 g respectively. The seed yield per plant ranged between 0.97 g and 10.07 g. Furthermore, estimation of seed β-ODAP content of 275 F3 genotypes were carried out, using modified protocol of Rao *et al*., 1964 [18] and Aletor *et al*., 1994 [4]. The mean seed β- ODAP content of segregating (F3) population was found to be 0.94% while the seed β-ODAP content of Mahateora and RLK-1950 were 0.06% and 6.15% respectively. The seed β-ODAP content ranged between 0.14% g and 3.84% respectively. Recombinant Inbreed lines *viz*. RIL no. 29, 120, 134 and 189 had less than 0.3% seed β-ODAP content. Of which, RIN no. 29 (4.36 g seed yield per plant) and RIL no. 189 (4.33g seed yield per plant) were considered as genotypes with medium seed yield per plant While, RIL no. 120 (6.23g seed yield per plant) and RIL no. 134 (6.13 g seed yield per plant) were considered as genotype with higher seed yield per plant.

The most variable quantitative trait was found to be the seed β-ODAP content, which was followed by seed yield per plant. While the lowest variability was found in biological yield per plant. Aksu *et al*., 2021 [21] reported that biological yield ranged from 2426.8 to 4222.3 kg ha-1 and seed yield ranges from 601.2 to 1430.7 kg ha-1 after evaluating twenty four *Lathyrus sativus* ICARDA lines with low β-ODAP content. Kosev and Vasileva *et al*., 2019 [12] also claimed that seed weight per plant ranged between 2.80 and 5.52 g. Bansara *et al*., 2016 [6] proclaimed that seed yield per plant ranged from 1142.4 to 2046.4 kg ha-1. Further, Polignano *et al*., 2005 [17] evaluated seventy six grass pea entries of different geographical origins and reported that entries showed a wide range of variation, seed yield and biomass being the most variable traits, evidence by coefficient of variation, where seed yield and biomass ranged between 7.0 to 214.0 and 13.0 to 481.0 g respectively. Mahapatra *et al*., 2020 [15] evaluated twenty different grass pea genotypes and observed that variability was highest in seed yield per plant, where the genotypes *viz*. IG-64842, IG- 114559, IFLA-1426, IG-65912 and Maharera were reported to be promising, considering the performance of genotypes for important yield attributing traits including seed yield per plant.

The mean, minimum and maximum values, standard deviation and coefficient of variation of four quantitative traits of segregating (F3) population are presented in the table 1. The frequency distribution graphs of biological yield per plant, pod yield per plant, seed yield per plant, seed pigmentation and seed β-ODAP content of each F3 line of segregating population are presented in the figure 2 (a), 2(b), 2(c), 2(d) and 2(e) respectively.

Table 1: Mean, minimum and maximum values, standard deviation and coefficient of variation of four quantitative traits of segregating (F3) population, Mahateora X RLK-1950.

Traits	Mean	Minimum	maximum	Standard Deviation (SD)	Coefficient of variation (C.V)
Biological yield per plant (g)	12.01	3.84	20.78	3.24	26.99
Pod yield per plant (g)	5.39	1.91	11.08	1.44	26.72
Seed yield per plant (g)	4.39	0.97	10.07	1.35	30.64
Seed β-ODAP content (%)	0.94	0.14	3.84	0.50	53.31

Biological yield per plant, pod yield per plant, seed yield per plant and seed β-ODAP content of Maharera were recorded as 17.20 g, 7.93 g, 5.64 g and 0.06% respectively. Similarly, Biological yield per plant, pod yield per plant, seed yield per plant and seed β-ODAP content of RLK-1950 were recorded as 20.2 g, 8.61 g, 3.33 g and 6.15% respectively.

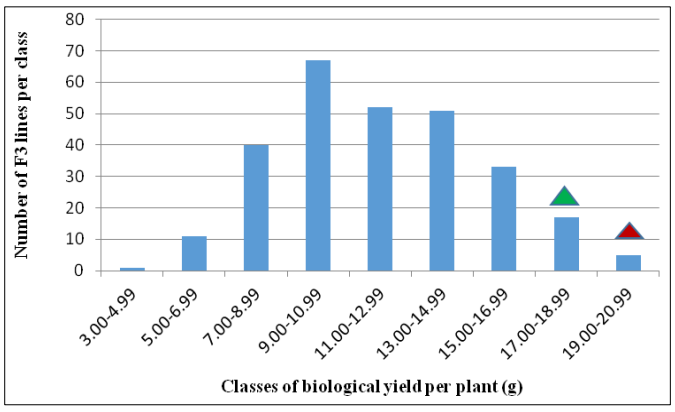

Fig 2(a): Frequency distribution graph of biological yield per plant of Segregating (F_3) population, Maharera X RLK-1950. Green triangle indicates Maharera value for trait and red triangle indicate RLK-1950 value for trait.

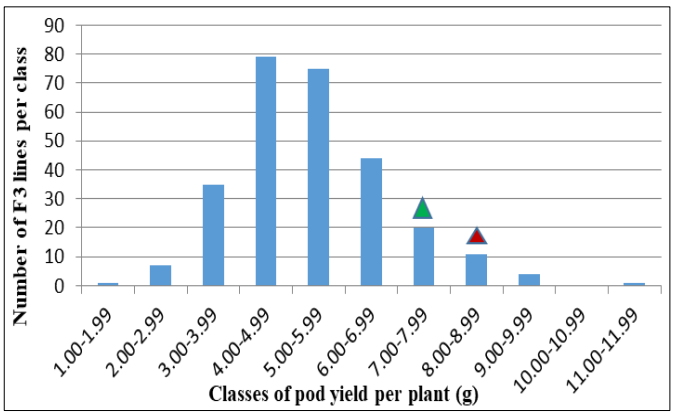

Fig 2(b): Frequency distribution graph of pod yield per plant of Segregating (F_3) population, Maharera X RLK-1950. Green triangle indicates Maharera value for trait and red triangle indicate RLK-1950 value for trait.

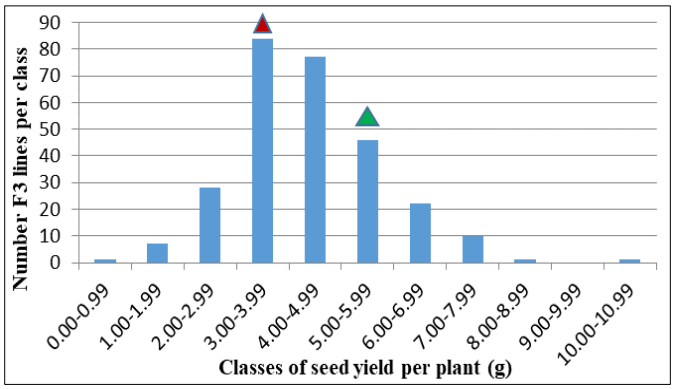

Fig 2(c): Frequency distribution graph of seed yield per plant of Segregating (F_3) population, Maharera X RLK-1950. Green triangle indicates Maharera value for trait and red triangle indicate RLK-1950 value for trait

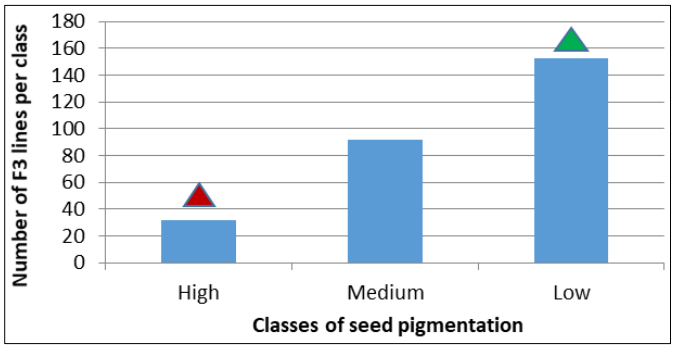

Fig 2(d): Frequency distribution graph of seed pigmentation of Segregating (F_3) population, Maharera X RLK-1950. Green triangle indicates Maharera value for trait and red triangle indicate RLK-1950 value for trait.

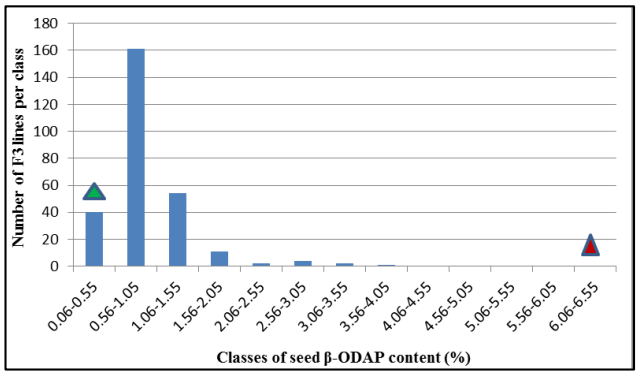

Fig 2(e): Frequency distribution graph of seed β-ODAP content of Segregating (F_3) population, Maharera X RLK-1950. Green triangle indicates Maharera value for trait and red triangle indicate RLK-1950 value for trait

3.2 Study of correlation between phenotypic traits and Seed β-ODAP content

The correlation between biological yields per plant, pod yield per plant, seed yield per plant as well as seed β-ODAP content was determined by Pearson's Correlation analysis. The resultant correlation coefficients (r) between the four quantitative traits *viz*. biological yield per plant, pod yield per plant, seed yield per plant and seed β-ODAP content are shown in Table 2. From the result, it is indicated that the biological yield per plant was positively as well as significantly correlated to pod yield per plant, seed yield per plant and seed β-ODAP content at $p \leq 0.01$. Similarly, pod yield per plant was positively as well as significantly correlated to biological yield per plant and seed yield per plant at $p \leq 0.01$ and significantly correlated to seed β-ODAP content at $p \leq 0.05$. Seed yield per plant was positively as well as significantly correlated to Biological yield per plant and pod yield per plant at $p \leq 0.01$ but it has positive non-significant correlation with seed β-ODAP content. Furthermore, seed β-ODAP content was positively as well as significantly correlated with biological yield per plant and pod yield per plant at $p \leq 0.01$ and at $p \leq 0.05$ respectively. However, seed β-ODAP content has positive but non-significant correlation with seed yield per plant. Aksu *et al.*, 2021 [21] reported that the seed yield was significantly and positively correlated to biologic yield (r = 0.759, $p<0.01$), which is similar to the results of this study. But pod yield was negatively correlated to seed yield and biological yield. Bansara *et al.* 2016 [6] reported that seed yield per plant was significantly and positively correlated with most of the investigated traits. Bansara *et al.* 2016 [6] reported that there was significant and positive correlation between ODAP content and seed yield. Kour *et al*, 2016 [13] reported that β-ODAP and seed yield were negatively or not significantly correlated, which is promising for development of high yielding low ODAP content varieties. However, in this study, β-ODAP content is positively correlated to seed yield per plant though it is non-significant.

Table 2: Correlation among four traits of *Lathyrus sativus* genotypes of segregating population

	BY	PY	SY	ODAP content
BY	1	0.574**	0.529**	0.157**
PY	0.574**	1	0.889**	0.143*
SY	0.529**	0.889**	1	0.082NS
ODAP content	0.157**	0.143*	0.082NS	1

BY-Biological yield per plant, PY-Pod yield per plant, SY-Seed yield per plant,
*- Significant at $p \leq 0.05$, **- Significant at $p \leq 0.01$

3.3 Selection of *Lathyrus sativus* genotypes considering high yield and low seed β- ODAP content

Many countries has considered *Lathyrus sativus* as promising to nourish an increasing population and to mitigate the challenge of climate change as it can be grown in stress prone adverse climatic condition.
(Hanbury *et al*., 1999) [9]. But the prolonged consumption of *Lathyrus sativus* seeds lead to the development of neurological disorder known as lathyrism, which is known to be caused by β-ODAP. (Moneim *et al*., 2001) [1]. Therefore, consumption of low ODAP content *Lathyrus sativus* seeds is preferable to high ODAP content seeds because *Lathyrus sativus* with no ODAP content is yet to be developed. Seed yield is considered as important trait while developing potential varieties of any crop, including *Lathyrus sativus*. Moreover, ODAP content is notably influenced by genotypes, environment as well as their interaction and many breeding programs have been initiated to combine low ODAP with high yield. As a result, high yielding varieties with low ODAP content have been developed in many countries. (Shiva Kumar *et al.* 2011) [14]. For instance, large scale evaluation has been undertaken in India, resulted in the development of high yielding low ODAP content varieties such as Maharera (0.074%), Prateek (0.076%) and Pusa 24 (0.2%). In addition to this, two high yielding low ODAP content varieties *viz* BARI Khesari-1 (0.06%) and BARI Khesari-2 (0.04%) have

been developed in Bangladesh. (Dahiya and Jeswani, 1974; Jeswani *et al.*, 1970; Somayajulu *et al.* 1975; Malek *et al.*, 1996) [7, 10, 21, 16]. After screening of 1128 accessions of cultivated *Lathyrus sativus* species, an extensive range of 0.150–0.952% for ODAP content was seen with only two low ODAP content accessions IG 118563 (0.150%) and IG 64888 (0.198%) (Abd-El-Moneim *et al.*, 2000) [2].

As a result of this study, Recombinant inbreed Line (RIL) no. 29, 120, 134, 189 and 216 had less than 0.3% seed β-ODAP content, which is considered as low but the seed yield per plant of RIL no. 216 was 3.81g, which is considered as low seed yield. However Yan *et al.*, 2006 confirmed that grass pea varieties with ODAP content less than 0.2% is safe for human consumption. Similarly, in this study RIL no. 29 has 0.17% seed β-ODAP content. RIL no. 120 and 134 had 6.23 g and 6.13 g seed yield per plant respectively, which are considered as higher seed yield, while RIL no.29 and 189 has medium seed yield per plant as 4.36 g and 4.33 g respectively, which are considered as medium seed yield. Hence, the RIL no. 29, 120, 134 and 189, identified from segregating (F_3) population, may be used for further generation advancement and development of low ODAP containing high yielding genotype. To establish the value of these genotypes, the leaf ODAP content at vegetative and reproductive growth stage have to be estimated. The genotypes with low leaf or seed ODAP content are potential candidates for *Lathyrus sativus* varieties development program. The seed β-ODAP content, seed yield per plant, pod yield per plant, biological yield per plant and seed pigmentation of RIL no. 29, 120, 134 and 189 are presented in the table 3.

Table 3: Seed β-ODAP content, seed yield per plant, pod yield per plant and biological yield per plant of selected four F3 lines, RIL no. 29, 120, 134 and 189.

RIL No.	Seed β-ODAP Content (%)	Seed yield per plant (g)	Pod yield per plant (g)	Biological yield per plant (g)	Seed pigmentation
29	0.17	4.36	4.63	15.17	Low
120	0.27	6.23	6.98	13.55	Low
134	0.28	6.13	8.18	14.97	Low
189	0.24	4.33	5.20	14.05	Low

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