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Identification of Toxic and Non-Toxic Lines in Jatropha

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A B S T R A C T

India spent Rs. 2,96,431 Crore to import 143.813 MMT of crude oil during April-November, 2016 showing a marked increase of 9.31% in quantity terms and 4.84% decrease in value terms over the same period of last year. Interspecific hybridization as prebreeding approach has been proved as an important approach to widen variability and potential plants emerged as derivatives have been effectively utilized in genetic improvement of Jatropha. The genetic approach proved applicable for utilizing nontoxic source applying conventional breeding and HPLC analysis of Phorbol 12-Myristate 13-Acetate (PMA) to develop nontoxic, high oil yielding lines of Jatropha. Analysis of plants comprising selected crosses involving Non-toxic lines, old crosses and backcrosses with and without non-toxic parents in early and late flowering, advanced line(s) and its clones on quantitative and qualitative traits viz. oil content and estimate PMA equivalent in seeds. PMA analysis by HPLC in seeds over the study material helped in identification of plants in crosses with low or no PMA and non-toxic (NT) plants from provenances. The detail analysis of PMA in seeds and its part precisely contributed in establishing the genetic basis (Maternal inheritance) and also hypothesizing involvement of more than one dominant genes controlling PMA content in Jatropha.

Introduction

Biofuels are discussed as one of the potential tools to establish linkage between development and environmental degradation. A large potential remains to be exploited in the sustainable production of first generation biofuels in developing countries. Efficiency considerations continue to indicate that feedstock and biofuel production can be done most favorably in developing countries, where the climate to grow them and low-cost farm labor continue to exist. *Jatropha curcas* L. a multipurpose large shrub is found throughout the tropical region belongs to family Euphorbiaceae. It is a plant with many attributes, multiple uses and considerable potential. It is a hardy plant, thriving on degraded land and requiring limited amounts of nutrients and water. It is widespread in many tropical and subtropical countries but not yet witnessed high yielding cultivars with proven yields through breeding and genetic interventions. Its seeds have been extensively investigated as a source of oil. The increasing availability of by-products from Jatropha oil production, their high protein content and, hence, their potential use as a feed material

has stimulated the development of various methods of extraction or degradation of Phorbol Ester present in Jatropha cake. *Jatropha curcas* biodiesel is 100% natural and bio degradable excited by the possibility of its wide-scale use in transport, electrical equipment and other machines that runs on diesel. Biodiesel from Jatropha can play a vital role in reducing the adverse effects of economic instability, greenhouse gases (GHGs) and helps in enhancing rural employment and development. Jatropha curcas has been found reasonably suitable due to high oil (18-38%), short gestation period, quality oil and other cogent reasons. Unknowningly, the consumptions caused mortality of infants and the limited use of oilcake due to toxicity followed development of detoxification methods. One kilogram of oil from Jatropha seeds generates about 0.75 kg of high quality cake with 65% protein (dry matter basis) with a favourable amino acid composition, but the toxic compounds "Phorbol" especially 12-myristate 13-acetate (PMA) highly toxic, limits its use in major Jatropha growing regions across the world. The oil from Jatropha can be converted into bio diesel by trans-esterification (Foidl *et al.,* 1996). Seed and vegetative part of Jatropha are toxic in nature. The toxic and non-toxic genotypes of *J. curcas* exist (Makkar and Becker 2009). It is also important to assess the antinutritional factors such as trypsin inhibitor and phytate (Makkar *et al.,* 2008, 2011), and protein digestibility such as pepsin insoluble nitrogen, pepsin plus trypsin digestibility and available lysine (Aderibigbe *et al.,* 1997, Makkar *et al.,* 1998). In addition to the antinutritional factors, the press cake from *J. curcas* has to be purified from its toxic phorbol esters, the main toxic component, before being used in feed production (Makkar and Becker 1999, Makkar *et al.,* 2008). Since the phorbol esters are also found in the oil (Devappa *et al.,* 2010, Makkar *et al.,* 2011), it is crucial to minimize the residual oil content in the press cake, in order to enhance the detoxification process. Makkar *et al.,* (1998) conducted comparative evaluation of non-toxic and toxic varieties of J. curcas for chemical composition, digestibility, protein degradability and toxic factors. Analyzed four varieties of J. curcas originated from Nicaragua (toxic varieties from Cape Verde and Nicaragua) Nigeria (toxic variety) and Mexico (Non-toxic variety). The occurrence of non-toxic lines its consumption as peanuts in Mexico attracted the attention. Makkar and Becker (2009) reviewed its potential for the production of biofuels, protein concentrates as livestock feed and value added products that could enhance the economic viability of Jatropha seed oil-based biodiesel producion.

Experimental material

Phorbol 12-Myristate 13-Acetate (PMA) analyzed in seeds in plant within crosses to validate the non-toxic lines using High Performance Liquid Chromatography (HPLC) analyzed by High Performance Thin Chromatography (HPTC). The PMA was also analyzed in seeds and all seed parts viz. Shell, kernel, tegmen, endosperm and cotyledon. The experiment comprises 199 plants selected amongst new and old crosses/backcrosses and clones of advanced lines:

Oil estimation

125 accessions based on sufficient high yielding ability were analyzed for oil content. The oil content in the sample was estimated by Soxhlet's extraction methods as described in A.O.A.C. (1980).

Phorbol ester analysis

Preparation of Sample

The seed samples were prepared following the protocol standardized by Makkar *et al.,*

(1998): 5 g of seeds and leaves of each plant were weighed. Ground with a small amount of sand using pestle and mortal then added 20ml of dichloromethane. The mixture was again ground for about 5 min with the morter.

The material is allowed to settle and the liquid was filtered and collected. Again the residue on the filter paper and in the pestle were pooled using about 20ml dichloromethane and then ground for 5 min. The filterate was again collected and it was repeated for 4-5 times. All the filtrate were pooled.

The residue (sand and kernels) was subjected to ultrasonic waves (105 W) for 3 min in 50 ml dichloromethane. It was then filtered and this filtrate was pooled with pooled previous extractions. The filtrate was dried at 40^0C under vacuum.

The dried residue was dissolved in 5ml tetrahydrofuran, passed through a filter and injected (10µl) into the HPLC.

Similarly, the samples were prepared from different seed parts viz. Shell, kernel, tegmen, endosperm and cotyledon in selected crosses. The procedure for separation used by Singh (2016) was followed and is given in brief. The kernels were removed pressing the hard shell (testa) by holding embryonic part of seed with thumb and first finger and other end between pliers; pressing gently cracked the testa. The splited testa was press open and detached to remove kernel.

The tegmen being very thin and tightly adhered with endosperm was removed by soaking the seeds in water for 6-8 hours. The soaked kernel was held flat with thumb and first finger and a light incision was given lengthwise on tegmen and tegmen was removed gently with rolling movement around endosperm. The cotyledon was removed by pressing two halves opposite the embryonic side to split open the endosperm, the visible cotyledon is gently lifted keeping the embryo intact, collected and dried in shed for analysis.

The quantity of tegmen and cotyledon was relatively low in volume and weight was used for preparing the samples similar to seed and kernel, however, the dried residue was added with tetrahydrofuran in proportion to the seed weight.

Preparation of Standard

The phorbol esters standard was prepared at the concentration of 1mg / 10 ml in methanol. 50µl of the standard was dissolved in 0.5 ml of methanol in a volumetric flask and used for the analysis.

HPLC Analysis of phorbol esters

The HPLC (WATERS) equipment used consist of W 600HPLC pump, 2996 photo diode array (PDA) detector, 717 auto sampler and the ODS_2 analytical column. Two solvent were used: (A) Water, (B) Acetonitrile in gradient mode. Solvent A was filtered before use; solvent B used was of HPLC and analytical grade and used after filtration.

All the solvents were degassed by ultrasonification and by application of vacuum. The gradient used was started with 50% A and 50% B, which remained same for next 5 minutes, increased A to 75% and decrease B to 25 % in the next 5 min, finally increase A to 100% and decrease B to 0 % in the next 5 min, and flow remain same (100 % A and 0 % B) for 2 minute. The separation was performed at room temperature (25^0 C) with the flow rate of 1.0 ml min^{-1}. The peak area for phorbol content was recorded. The peaks were integrated at 280 nm with highest sensitivity and the retention time (RT -10.099) minute was recorded as equivalent to Phorbol-12 Myristate 13 Acetate.

Results and Discussion

Qualitative Traits

Phorbol 12-Myristate 13-Acetate (PMA)

PMA analysis was performed in seeds over plants in new and old crosses (70), plants from provenances (7) in seed, kernel and cotyledon and hybrid seeds in four direct and reciprocal crosses and parents in seeds and different parts of seeds viz. Shell, Kernel, Tegmen, Endosperm and Cotyledon by HPLC. The estimates by HPTLC (Singh, 2016) were compared with the estimates of HPLC analyzed under the study. The PMA estimate in the classes of material is given in Tables (2 to 5.).

Twenty nine plants in new crosses were analyzed for PMA by HPLC under the present study (Table 2.). Seventeen out of 29 were analyzed by HPLC for the first time; where eleven depicted non detection. The range of PMA values varied from non-detection to 0.04300 mg/g of seed. The cross 45(34)26 was observed to posses 0.04300 mg/g of PMA which is close to the toxic value of 0.05000. All plants were classified as non-toxic. The estimates of twelve plants analyzed by HPTLC exhibited non detection. The overall status of thirteen plants cited in bold are classified as non-toxic with high accuracy of estimation.

Forty one plants comprised 36 plants from old crosses and five plants as parents were analyzed for PMA by HPLC under the present study (Table3.). One plant out of five plants exhibited PMA between ND to 0.01300; remaining four derived from same cross exhibited a range between 0.00038 to 0.01300. One plant Fourteen out of 36 were analyzed by HPLC for the first time; where, only eleven depicted non detection rest other with PMA varying between ND to as high as 0.02800. All plants were classified as non-toxic by HPLC analysis. The estimates of in eight plants out of 24 analyzed by HPTLC exhibited non detection similar to HPLC rest exhibited presence of PMA below the toxic limits. The overall status of the analysis of 41 plants only seven plants cited in bold with non-detection are classified as non-toxic with high accuracy of estimation.

The plants at serial no. 9-11, 18-20, 24 and 25, 29 and 30, 34-37, derived from same set of crosses showed variation in PMA estimates involving NT parent (*J. curcas*)used once, either as female or male parent alone or in combination. Similarly the plants at serial no. 38 to 40 not involving NT parent also exhibited variation which involves *J. integerimma* as parent.

Two plants each from provenance NBJ-1, MP-55, PDKV and one plant from RJ 127 was analyzed to confirm toxicity of plants analyzed earlier (Singh 2015) by HPTLC. The PMA was estimated in Seeds, Kernel and Cotyledon to know the extent of presence.

The PMA estimates in seeds of plants in both the plants of NBJ1-1 and NBJ1-2 exhibited 0.00590 and 0.00420 mg/g of seed respectively. The PMA in kernel and cotyledon in NBJ1-1 was not detectable however, NBJ1-2 exhibited 0.00015 and 0.00090 mg/g. Both the plants were identified as non-toxic. The plants in provenance MP55-1 and MP55-2 expressed estimates to the extent of 0.14570 and 0.00630 mg/g in seeds respectively. The PMA estimates in kernel and cotyledon in MP55-2 and cotyledon in plant MP55-1 was not detected whereas, kernel borne 0.00012 mg/g of seed (Table 4.).

The PMA estimates in seeds of plants provenance PDKV 1 and 2 borne 0.08000 and 0.00430 mg/g of seed are in the range of toxic and non-toxic category respectively.

Table.1 Details of experimental materials

S. No.	Code	S. No.	Code	S. No.	Code	S. No.	Code	S. No.	Code	S. No.	Code
1	31(09)08	35	29(08)03	69	18(21)06	103	G4-7	137	H1-5-5-3	171	C34-6-4
2	34(18)01	36	32(03)01	70	19(23)15	104	H2-12	138	H1-5-5-5	172	C34-6-5
3	34(18)05	37	33(08)01	71	19(23)21	105	H3-12	139	H1-5-5-6	173	C34-6-6
4	34(18)06	38	34(18)10	72	23(01)01	106	H3-14	140	H1-5-5-7	174	C34-6-7
5	34(18)08	39	34(18)11	73	25(10)01	107	H4-1	141	H1-5-5-9	175	C34-6-8
6	34(18)12	40	36(15)07	74	25(10)02	108	H4-15	142	H-9-14-3-3	176	C34-6-9
7	34(18)16	41	38(15)11	75	25(10)03	109	H5-1	143	H9-14-3-4	177	C34-6-10
8	34(18)18	42	39(34)06	76	25(10)05	110	H5-9	144	H9-14-4-9	178	E9-7-1C
9	35(04)01	43	39(34)09	77	26(18)08	111	H5-15	145	G1-8-2-6	179	E9-7-2C
10	36(15)01	44	39(34)13	78	A1-5	112	H6-1	146	G1-8-3-3	180	E9-7-3C
11	36(15)03	45	39(34)15	79	A7-2	113	H6-7	147	H1-6-2-4	181	E9-7-4C
12	36(15)14	46	39(34)16	80	B3-11	114	H6-12	148	H1-6-2-6	182	E9-7-5C
13	36(15)15	47	39(34)18	81	B5-3	115	H7-14	149	H1-6-2-10	183	E9-7-6C
14	38(15)03	48	39(34)21	82	B6-10	116	H8-1	150	H1-6-3-2	184	E9-7-7C
15	39(34)11	49	39(34)24	83	B8-1	117	H8-2	151	H1-6-3-3	185	E9-7-8C
16	39(34)27	50	39(34)31	84	B8-2	118	H8-3	152	H1-6-3-10	186	E9-7-9C
17	39(34)29	51	39(34)32	85	B8-5	119	H9-2	153	H1-2-4-9	187	E9-7-10C
18	41(25)06	52	41(25)01	86	B8-7	120	H9-7	154	H4-15-2-1	188	E9-7-11C
19	41(25)07	53	41(25)02	87	B8-10	121	H9-9	155	H4-15-5-10	189	E9-7-12C
20	41(25)13	54	41(25)09	88	B8-13	122	H10-14	156	H6-7-3-1	190	E9-7-13C
21	41(25)16	55	41(25)12	89	B9-8	123	I10-1	157	H3-12-3-8	191	E9-7-14C
22	42(20)12	56	42(20)01	90	B9-14	124	I12-1	158	H-9-9-2-4	192	T1
23	45(34)08	57	42(20)02	91	D1-6	125	I13-3	159	61-3	193	T2
24	45(34)17	58	42(20)07	92	E1-2	126	I14-4	160	52-2	194	T3
25	45(34)24	59	42(20)10	93	E1-4	127	G3-3-4-5	161	52-3	195	T4
26	45(34)26	60	42(20)20	94	E1-6	128	G3-3-4-6	162	52-5	196	T5
27	46(04)04	61	43(03)01	95	E1-8	129	H2-1-2-4	163	H10-4	197	T6
28	01(18)01	62	44(08)04	96	E9-7	130	H2-1-2-8	164	H10-5	198	T7
29	03(05)01	63	45(34)06	97	G1-6	131	H2-1-3-10	165	H10-6	199	T8
30	08(24)21	64	47(01)01	98	G1-8	132	H2-1-4-4	166	H10-7		
31	15(02)01	65	48(05)04	99	G2-5	133	H2-1-4-7	167	H10-8		
32	19(23)20	66	02(04)04	100	G2-7	134	H2-1-5-7	168	C34-6-1		
33	26(18)03	67	16(18)07	101	G2-8	135	H2-1-5-9	169	C34-6-2		
34	29(08)01	68	16(18)12	102	G3-8	136	H1-5-4-4	170	C34-6-3		

Table.2 PMA estimates analyzed by HPLC and its comparison with HPTLC in Selected plants amongst New Crosses

S.no	Code	PMA mg/g of Seed	Classified as	Overall status

		HPTLC	Classified as	HPLC		
1	32 (03)01	-	-	NA	NT	NT
2	34(18)01	Nd	NT	ND	NT	NT
3	34 (18)03	-	-	0.00250	NT	NT
4	34 (18)05	ND	NT	0.00830	NT	NT
5	34 (18)04	ND	NT	0.00094	NT	NT
6	34(18)06	ND	NT	0.00066	NT	NT
7	34(18)08	ND	NT	0.00055	NT	NT
8	34(18)14	ND	NT	0.00061	NT	NT
9	34(18)18	ND	NT	0.00045	NT	NT
10	35(04)01	-	-	0.00250	NT	NT
11	36(15)01	ND	NT	NA	NT	NT
12	36(15)03	-		NA	NT	NT
13	36(15)07	-	-	0.00035	NT	NT
14	38(15)03	ND	NT	NA	NT	NT
15	39(34)09	ND	NT	0.00230	NT	NT
16	39(34)13	-	-	NA	NT	NT
17	39(34)31	-		0.03800	NT	NT
18	41(25)01	ND	NT	NA	NT	NT
19	41(25)09	-	-	0.00310	NT	NT
20	42(20)09	-	-	0.00310	NT	NT
21	45(34)15	-	-	0.00280	NT	NT
22	45(34)26	ND	NT	0.04300	NT	NT
23	18(21)06	-	-	0.00160	NT	NT
24	19(23)15	-	-	0.00061	NT	NT
25	11(07)10	-	-	0.00300	NT	NT
26	19(23)21	-	-	ND	NT	NT
27	19(23)15	-	-	ND	NT	NT
28	22(09)04	-	-	ND	NT	NT
29	19(23)16	-	-	ND	NT	NT

Table.3 Comparison of PMA estimates by HPTLC and HPLC in Parents and old crosses

S. No.	Code	PMA mg/g of Seed	Overall

		HPTLC	Classified as	HPLC	Classified as	Status
1	61-3	ND	**NT**	ND	**NT**	**NT**
2	52-2	ND	**NT**	0.00038	NT	NT
3	52-3	ND	**NT**	0.00150	NT	NT
4	52-5	-	-	0.01300	NT	NT
5	A1-5	0.0108	NT	NA	NT	NT
6	A4-4	-	-	0.02200	NT	NT
7	A7-2	ND	**NT**	ND	**NT**	**NT**
8	A9-1	-	-	0.00039	NT	NT
9	B8-1	-	-	0.00228	NT	NT
10	B8-5	-	-	0.00154	NT	NT
11	B8-7	-	-	0.00379	NT	NT
12	B9-14	-	-	0.02500	NT	NT
13	C34--6			NA	NT	NT
14	E9-7	ND	NT	NA	NT	NT
15	G1-6	-	-	0.00314	NT	NT
16	G1-8	-	-	0.00700	NT	NT
17	G2-3	ND	**NT**	0.00110	NT	NT
18	G2-5	ND	**NT**	0.00190	NT	NT
19	G2-6	ND	**NT**	0.00246	NT	NT
20	G2-7	0.0068	NT	0.00590	NT	NT
21	G3-3	ND	**NT**	0.02800	NT	NT
22	G4-7	-	-	0.00440	NT	NT
23	H1-2	ND	**NT**	0.00410	NT	NT
24	H1-5	0.0344	NT	0.00037	NT	NT
25	H1-6	0.0284	NT	0.00500	NT	NT
26	H2-1	0.0704	NT	0.00590	NT	NT
27	H3-12	ND	**NT**	ND	**NT**	**NT**
28	H3-14	ND	**NT**	ND	**NT**	**NT**
29	H4-1	-	-	0.01700	NT	NT
30	H4-15	0.0637	NT	0.00093	NT	NT
31	H5-1	ND	**NT**	ND	**NT**	**NT**
32	H6-7	ND	**NT**	ND	**NT**	**NT**
33	H9-7	-	-	0.00790	NT	NT
34	H9-9	-	-	NA	NT	NT
35	H9-14	0.0208	NT	0.00100	NT	NT
36	H10-14	-	-	0.0022	NT	NT
37	I10-1	ND	**NT**	0.0058	NT	NT
38	I12-1	ND	**NT**	0.0267	NT	NT
39	I13-3	-	-	0.0220	NT	NT
40	I14-4	NA	NT	0.0400	NT	NT

Table.4 PMA estimates in Seed, Kernel and Cotyledon in Selected plants among Provenances by HPLC

Genotype	Seed and Parts	PMA mg/g of Seed	Classification	Overall Status Seed Basis
NBJ1-1	Seed	0.00590	NT	Non-toxic
	Kernel	ND	NT	
	Cotyledon	ND	NT	
NBJ-1-2	Seed	0.00420	NT	Non-toxic
	Kernel	0.00015	NT	
	Cotyledon	0.00090	NT	
MP-55-1	Seed	0.14570	**T**	Toxic
	Kernel	0.00012	NT	
	Cotyledon	ND	NT	
MP55-2	Seed	0.00630	NT	Non-toxic
	Kernel	ND	NT	
	Cotyledon	ND	NT	
PDKV-1	Seed	0.08000	**T**	Toxic
	Kernel	0.00040	NT	
	Cotyledon	0.00037	NT	
PDKV-2	Seed	0.00430	NT	Non-toxic
	Kernel	0.00190	NT	
	Cotyledon	0.00016	NT	
RJ127-2	Seed	0.00392	NT	Non-toxic
	Kernel	0.00011	NT	
	Cotyledon	0.00015	NT	

Table.5 Comparative estimates of PMA content (mg/g) analyzed by HPTLC and HPLC over seed, shell, kernel, tegmen, endosperm and cotyledon in direct, reciprocal crosses and parents

Parents and Crosses	Method	Seed	Shell	Kernel	Tegmen	Endosperm	Cotyledon	Overall Status
MP55-1 (T)	HPTLC	0.0559	ND	ND	0.4090	ND	ND	Toxic
	HPLC	0.0530	-	-	0.1900	-	0.0007	Toxic
MP55-1×61-3	HPTLC	NA	ND	0.0576	ND	0.0673	ND	Toxic
	HPLC	-	-	NA	0.0023	NA	0.0035	Non-toxic
61-3×MP55-1	HPTLC	NA	ND	NA	ND	0.0580	NA	Toxic
	HPLC	-	0.0079	-	-	0.1300	-	Toxic
61-3 (NT)	HPTLC	ND	ND	NA	ND	ND	NA	Non-toxic
	HPLC	-	0.0260	-	0.0019	-	-	Non-toxic
MP55-2 (NT)	HPTLC	ND	ND	ND	ND	ND	ND	Non-toxic
	HPLC	0.0030	NA	-	0.0094	-	NA	Non-toxic
MP55-2×61-3	HPTLC	ND	ND	ND	ND	ND	ND	Non-toxic
	HPLC	0.0049	NA	NA	NA	NA	NA	Non-toxic
61-3XMP55-2	HPTLC	ND	ND	NA	1.1009	ND	NA	Toxic
	HPLC	-	-	-	NA	NA		Non-toxic
61-3 (NT)	HPTLC	ND	ND	NA	ND	ND	NA	Non-toxic
	HPLC	-	0.0260	-	0.0019	-	-	Non-toxic

The PMA estimates in kernel and cotyledon in both the plants are below the range of toxicity. The PMA estimates in seed, kernel and cotyledon in plant in provenance RJ 127-2 exhibited 0.00392, 0.00011 and 0.00015 is in non-toxic range (Table 4.).

It is pertinent to mention that the PMA estimates by HPLC in seeds precisely classify two plants viz. MP55-1 and MP55-2 as toxic and non-toxic respectively (Table 4.) and PMA-Seeds in seeds of 61-3 is identified as non-toxic (ND). The analysis in seeds (Table 5.) by HPLC exhibited 0.0530 mg/g in P1 (MP 55-1) whereas; P2 (62-3) registered 0.0260 mg/g of PMA. PMA-Tegmen in P1 registered a value of 0.1900 and cotyledon with lower quantity (0.0007 mg/g) and no detection in shell kernel and endosperm; whereas, P2-Tegmen observed to have 0.0019 mg/g. The PMA-Kernel and endosperm in first cross MP55-1X61-3 was not detected by the system (NA) and tegmen and cotyledon borne 0.0023 and 0.0035 mg/g respectively, while its reciprocal 61-3 X MP55-1 showed PMA in shell (0.0079) and endosperm (0.1300). The PMA content in seed and tegmen was recorded as 0.0030 and 0.0094 respectively whereas; no PMA was recorded in shell and cotyledon. The second cross MP55-2x61-3 exhibited 0.0049 mg/g of PMA in seeds whereas; system didn't record PMA in shell, kernel, tegmen, endosperm and cotyledon. The PMA was not observed in tegmen of a reciprocal cross 61-3xMP55-2. The PMA content in shell exhibited ND/NA revealing its absence in all the treatments. The nontoxic line 61-3 consistently expressed absence of PMA in all the parts.

Phorbol estimation

The information cited in review for phorbol analysis mainly reveal identification of non-toxic lines by HPLC and HPTLC (Makkar and Becker 1997, Becker and Makkar 1998, Makkar *et al.*, 1998, Makkar and Becker 2009), also classified by using molecular tools (He *et al.*, 2011, King *et al.*, 2013) but this study mainly concentrate on identification and utilization of NT source in hybridization programme and identification of promising NT lines for use in the developmental programme.

The Phorbol 12- Myristate 13- Acetate (PMA) in seeds over five classes of material involving 52-2 and 61-3 as nontoxic parent involved in various cross were analyzed using HPLC. The plants amongst the crosses with seed yield above 500g per plant were isolated for PMA analysis. The estimates of phorbol over seeds and its parts are discussed below.

PMA estimates in New Crosses involving NT as female and male parent

All the twenty two crosses exhibited absence of PMA and observed as not detected (ND) were classified as nontoxic (NT). Thirteen plants in crosses involving 61-3 as female parent was classified as nontoxic. The involvement of 61-3 and 52-2 as donor of NT trait expressed whether used as female or male parent in single and two way crosses also resulted in nontoxic plants.

PMA estimates in New Crosses without NT parent

All the seven crosses in new breeding block not involving the NT source exhibited non detection of PMA or very low value (0.00061) in cross 14-22×Dwarf×Dwarf. The involvement of *J. integerimma* in prebreeding programme extended dual advantage of delivering vast variability for all traits including non-toxic gene clearly reveals that this ornamental type is the donor of nontoxic gene in other crosses not involving 61-3 or 52-2. Therefore, the initial advance line viz. C34-6 derived from interspecific cross and

backcross to *J. curcas* involved NT gene, turned out to be non-toxic with no detection or very minor content.

PMA estimates in backcrosses with and without NT parent

All the forty one crosses amongst the backcross population (Old) analyzed for PMA with and without NT parent were also classified as nontoxic. The plants at serial no. 9-11, 18-20, 24 and 25, 29 and 30, 34-37, cited in Table 3 are derived from same set of crosses showed variation in PMA estimates involving NT parent (*J. curcas*) used once, either as female or male parent alone or in combination resulted into nontoxic type. The reason for the source of NT gene in pre-bred material confirms its presence in ornamental jatropha species *J. integerimma.* Similarly the plants at serial no. 38 to 40 not involving NT parent also exhibited variation which involves *J. integerimma* as parent.

PMA estimates in selected plant among provenances

Analysis of PMA in seeds of plants among four provenances helped categorize indigenous collections as toxic and non-toxic. Both the plants in NBJ 1-1 and NBJ1-2 were identified as Non-toxic. One plant each in MP 55 and PDKV viz. MP55-2 and PDKV -2 were identified as Non-toxic and rest two as toxic. The only plant in RJ 127-2 was also identified as non-toxic. The identification of indigenous NT source will be an add advantage of adaptation utilization in breeding programme.

PMA estimates in parents, direct and reciprocal crosses

The Analysis of PMA in seeds and different parts of seed in direct and reciprocal crosses facilitated understanding its presence or absence and presence in quantitative terms. The PMA is absent in shell subject to the condition that no part of tegmen remain stick to the inside of shell. The separation of tegmen with the removal of shell and precise peeling of tegmen from cotyledon confirmed its presence in maternal tissue the Tegmen confirms maternal inheritance with dominant effect and no or traces quantity is observed in cotyledon. Both 61-3 and 52-2 used as donor of gene in direct and reciprocal crosses expressed very effectively, as not detected or variable quantity below the toxic limit. The cross MP55-1×61-3 where female parent is toxic strongly proved its presence in Tegmen confirmed maternal inheritance; similarly its reciprocal cross 61-3×MP 55-1 exhibited traces of PMA below toxic limit also confirmed maternal inheritance. The vide variation for PMA across crosses under study also indicate involvement of more than one dominant gene governing toxicity. Another cross MP55-2×61-3 exhibited 0.0049 mg/g of PMA in seeds where MP55-2 is nontoxic; system didn't record PMA in any part of seed and its reciprocal cross 61-3×MP55-2, PMA was not observed in tegmen confirms both the parents non-toxic. It is also concluded that HPTLC facilitate rapid screening of material for PMA. The analysis of PMA in tegmen alone may be tried by HPTLC for broad classification and precise quantitative estimates could be done later by HPLC. The values in HPLC estimates reveal precision of analysis by HPLC as compared to HPTLC.

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