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Full Length Research Paper

Development and screening of cowpea recombinant inbred lines for seedling drought tolerance

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The use of multiple traits for drought study affirms the complexity of drought tolerance in cowpea. Despite the availability of several traits for drought tolerance evaluation, the rapid screening technique used by many scientists for seedling drought in wooden boxes is the simplest method for screening large populations. The objective of this study was to select drought tolerant cowpea recombinant lines developed from a drought tolerant and susceptible parent using the wooden box screening technique. Two hundred Recombinant Inbred Lines (RILs) an $F_{2:6}$ generation were used for the study. The parents were drought tolerant line crossed with susceptible line. Screening was done in wooden boxes and plants stressed for 4 weeks and in two sessions. Leaf wilting, relative water content, chlorophyll content during stress, and recovery from drought data were taken. Results from this study showed significant differences ($p < 0.05$) for relative chlorophyll content for the 4 weeks of water stress and relative water content taken on the second week of water stress for all 200 inbred lines, but no significant differences were observed for the parental checks. Relative water contents taken for RILs during water-stress ranged between 70-20% for drought tolerant and drought susceptible lines respectively. The parental lines used as checks both had relative water contents of 60%. Relative water content for the second experiment ranged between 74-22% for tolerant and susceptible RILs respectively. About 12 inbred lines consistently performed well for recovery, 13 RILs were susceptible. RILs that maintained a higher relative water and chlorophyll contents, with high proportion of survived seedlings were 11. Potential seedling drought tolerant RILs have been identified.

Key words: Cowpea, leaf wilting, chlorophyll, recombinant inbred lines, drought tolerance, relative water content.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] being an excellent source of protein, contains all the essential amino acids (Vasconcelos et al., 2010; Oliveira et al., 2016). It is

also rich in carbohydrates, vitamins and minerals, besides having great fibre content and low-fat content, constituting an important food component in several

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countries including Ghana (Freire Filho et al., 2012; SARI, 2014). As such the crop is grown in all the Savanna ecological regions of Ghana where drought and heat stress compounded by poor soil fertility conditions limit the production of many other crops. Among the abiotic constraints to production of the crop, drought is one of the most important stresses because, the crop is typically grown in the Savanna and Sahel regions where rainfall amount and distribution are unreliable. The severity and occurrence of drought stress is expected to increase as a result of global environmental changes, causing major implications for food supply (Fan et al., 2015). Compounding this is an increasing world population that requires a rise in food production by more than 70% before 2050 (Godfray et al., 2010; Parfitt et al., 2010; Wallace, 2000).

Verbree et al. (2015) indicated that, the use of multiple traits for evaluation of drought tolerance affirms the complexity of drought tolerance in cowpeas compared to other crops. Also, Aliyu and Makinde (2016) and Swain et al. (2017) indicated that cowpea breeding is largely based on selection of parents, followed by hybridization, in order to form a base population and generation advancement with simultaneous selection for more than one trait (Batiemo et al., 2016). Therefore, the most common breeding method consists of screening under controlled drought stress; the offspring derived from populations, followed by the assessment of selected genotypes at a location where drought occurs frequently, and testing the most promising genotypes for yield potential and yield stability in multiple sites representing the target ecology (Batiemo et al., 2016; Ortiz et al., 2002). Analysis of genetic divergence seeks to identify parents for creating populations with genetic variability and consequent genetic gain in successive selection cycles (Santos et al., 2016). Though various cowpea-breeding materials such as F₂, F₃ and backcross populations have been used for drought tolerance studies in cowpea, the empirical approach mainly relies on the use of recombinant inbred lines (RIL) to enable the consistent evaluation of performance and understanding of genotype-by-environment interaction, as the intensity and frequency of naturally occurring drought stress are not predictable. The RIL population, developed through single seed descent of several selfed generations consists of individual lines carrying dispersed homozygous segments of a parental chromosome. The objective of this study was to select drought tolerant cowpea recombinant lines developed from a drought tolerant and susceptible parents using the wooden box screening technique.

MATERIALS AND METHODS

Population development

Four hundred and fifty Recombinant Inbred Lines (RILs) were

developed through single seed descent and an F_{2:6} generation was obtained between 2010 and 2015. The parents for the developed population were IT-93k-503-1; a drought tolerant and a medium maturing, indeterminate line crossed with IT97k-279-3; an early maturing line with determinate character. These two lines were obtained from the International Institute of Tropical Agriculture (IITA) Kano, Nigeria.

Geographical location and experimental design for screen-house experiment

The study was carried out at Savanna Agricultural Research Institute (SARI), Nyankpala. Nyankpala is located in the Northern Guinea Savanna Zone with a mean annual rainfall of about 1000 mm. It is located on latitude 9°, 25" N and longitude 0°, 58" W with an altitude of 183 m above sea level. Wooden boxes of 130 cm length, 65 cm width, 15 cm depth, and 2.5 cm thick planks were arranged in a screen house as described by Singh et al. (1999a). The boxes were lined with perforated polyethylene sheets and filled with one: one mixture of top soil and sand which averaged 39% sand, 2% clay, and 59% silt by analysis of the composited soil used to fill the boxes. The composite soil had a pH value of 6 and an organic matter content of 6% with N, P, K of 0.05, 3, and 45% respectively. The soil had a bulk density of 1.33 g/cm³. *Bulk density (g/cm³) was manually estimated as: Dry soil weight (g) / Soil volume (cm³)* The boxes were filled to 12 cm depth leaving about 3 cm space on the top for watering. The polyethylene lining along the sides and bottom of the boxes ensured even distribution of water. A spirit level was used to ensure a flat soil surface on the boxes before and after watering. Equidistant holes were made in straight rows 10 cm apart with a hill to hill distance of 5 cm within the rows. Each box was watered thoroughly and allowed to drain for two days before planting. The moisture content was then taken before planting using the WET sensor with the HH2 moisture meter (Plate 3). Two seeds were sown in each hole and were thinned to one plant per hill one week after germination. Each box contained one row each of 10 recombinant inbred lines plus the two parental checks making it 12 lines in each box (Table 1.). Seedling drought screening was done in two sessions using augmented design because of large size of recombinant inbred lines to be tested (200), as well as limited space for experiment arrangement in the screening house. Screen house experiment one (session one) was done between June and July 2015, while screen house experiment two (session two) was done between October and November 2015, as a repeat to confirm seedling screening for drought tolerance. The boxes were watered daily using a small watering can until the appearance of the first trifoliate leaf, after which watering was stopped (Plate 1 and 2).

Climatic data for the period of the screen-house experiment

The mean average temperature within the screen house during the period of the experiment for the two sessions ranged between 26.4 and 30.7°C, similarly, the mean relative humidity ranged between 47 and 83%.

Drought treatment

Moisture stress was applied by watering the plants until the full expansion of the first trifoliate leaves (two weeks after planting), after which watering was withdrawn for four weeks, in order to take drought response measurements (Muchero et al., 2008). The plants were then re-watered twice a week for a period of two weeks, before taking recovery measurements. The SM300 soil moisture meters with the HH2 reader manufactured by the DELTA-T Devices Ltd, UK, was used together to monitor the soil moisture

Table 1. Arrangement of recombinant inbred lines in boxes for Screen-house experiment.

Boxes																			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
84	300	319	46	11	385	384	229	22	350	111	142	309	326	309	333	403	171	130	70
40	343	75	306	92	37	255	15	398	194	222	282	39	200	231	404	238	96	157	279
179	228	186	136	390	187	352	167	245	258	5	55	297	312	378	25	332	340	192	62
195	178	3	161	314	325	281	225	124	361	407	232	301	362	19	134	294	54	318	211
263	45	158	76	240	169	268	101	182	58	372	356	401	365	88	72	406	230	121	243
338	261	256	20	316	116	4	351	66	242	162	202	360	175	341	320	346	38	310	149
30	212	10	325	246	190	17	99	419	28	29	193	416	353	249	410	223	376	7	413
164	253	131	284	221	112	82	156	210	135	272	189	382	348	241	57	61	90	91	2
13	87	94	17	233	283	78	140	408	235	43	234	122	64	133	119	308	342	409	73
106	209	321	373	396	405	307	47	6	292	286	26	418	9	27	368	197	137	298	260
Standards																			
IT93K-503-1																			
IT97K-279-3																			



1



2

Plate 1 and 2. Emergence and appearance of trifoliate leaves.

directly in the soil on a weekly basis during the water stress imposition until the end of the experiment (Plate 3).

Weekly chlorophyll meter readings

Soil Plant Analytical Development (SPAD) chlorophyll meter reading was taken at a weekly interval from the first week of the experiment until the end of the experiment. The Minolta handheld portable SCMR meter (SPAD- 502 Minolta, Tokyo, Japan), was used as per Markwell et al. (1995) to acquire a rapid estimate of the leaf chlorophyll content in nmol/cm. The measurements were taken on the upper most collared leaf halfway from the leaf base to the tip and halfway from the midrib to the leaf margin. Four measurements were taken per plant and the results averaged resulting in a single value to represent each inbred line. In recording the Specific chlorophyll metre readings (SCMR), care was taken to ensure that

the SPAD meter sensor fully covered the leaf lamina and the interference from veins and midribs were avoided.

Leaf wilting

Leaf wilting index were calculated from the first week of stress to the final week using Mai-Kodomi (MAIK) scale, by Mai-Kodomi et al. (1999); total number of leaves per plant; number of leaves showing wilting signs with the following wilting scale: 0 = no sign of wilting, 1 = 25% of wilting 2 = moderate wilting, 50%, 3 = yellow and brown leaves with 75% wilting, 4 = completely wilted.

Relative water content measurements (RWC)

Relative water content (RWC) was calculated on new fully



Plate 3. moisture determination using the WET sensor to monitor moisture levels during experiment.

expanded leaflets after the second and fourth weeks of stress, as outlined in Bogale et al. (2011). The leaves for RWC were detached from the plant between 10 am and 2 pm during bright days, in order to avoid the effects of weather conditions on water loss from the detached leaves. Immediately after cutting at the base of the lamina, the leaves were weighed to obtain the fresh weight (FW). After weighing, the leaves were soaked in deionized water for 48 hours at room temperature for rehydration: and then re-weighed for turgid weight (TW). The leaves were then dried in an oven at 70°C for 72 h before dry weight (DW) measurements were taken. The RWC was calculated as follows:

$$RWC = \frac{FW - DW}{TW - DW} \times 100\%$$

(Bogale et al., 2011).

Visual vigour rating of seedlings under water stress

The following parameters were recorded after stressing the plants: wilting, using Mai-Kodomi (MAIK) (Mai-Kodomi et al. 1999) scales: total number of leaves per plant; number of leaves showing wilting signs per plant; and RWC. The Leaf Wilting Index (LWI) were calculated weekly, from the first week to the final week of stress, as the ratio between leaves showing wilting signs and the total number of leaves per plant. Both the IB and MAIK scales were scored on a weekly basis from the second week until the end of the stress period.

Recovery from drought

After re-watering, data were collected on: Survival count (SC): number of surviving plants per genotype.

Recovery rate (RR)

Recovery rate was computed as:

$$\frac{\text{Proportion of survived plants}}{\text{Total no of emerged plants}} \times 100$$

(Fatokun et al. 2012)

Plate 4 shows the reaction of inbred lines to water stress treatment up to the fourth week, followed by recovery after watering resumed (Plate 5 and 6).

Data analysis

Data was analysed using GenStat edition version 12, and SAS (version 9.4; SAS Institute, Cary NC). Phenotypic correlation and regression analysis were then performed using PROC CORR and PROC REG to determine the association among the physiological parameters. Significant means were separated using the least significant difference at 5% probability level (LSD_{0.05}).

RESULTS

Variation in seedling stage drought tolerance based on box screening technique

Chlorophyll content

There were variations among the inbred lines screened for drought in the screen-house for both screen-house experiments one and two. The chlorophyll content in nmol/mg gradually decreased over the period of the stress imposition with chlorophyll content ranging between 40.49 and 28.89 with the parental checks of 35.68 and 36.30 for IT93K- 503-1 and IT97K-279-3 respectively for week one and 32.93 and 9.03 with the parental checks recording 25.26 and 23.50 for week four of stress for screen-house experiment one. Chlorophyll measurements for screen-house two showed similar pattern of variation in terms of reduction in chlorophyll as the stress imposition advanced. The chlorophyll for week one ranged between 46.72 and 38.52 with the parental checks recording values in the range of 39.56 and 35.43; week two values ranged between 42.11 and 30.97 with



Plate 4. Response in inbred lines to moisture stress at four weeks.



5



6

Plate 5 and 6. Recovery from seedling drought screening in the screen house.

parental values of 38.56 and 33.45 for IT93K-503-1 and IT97K-279-3 respectively. Also, leaf chlorophyll content for week four ranged between 29.7 and 9.00 with the parental checks ranging between 18.25 and 25.85.

Relative water content for screen-house experiment one and experiment two

The relative water contents ranged between 20 and 70% for both drought susceptible and drought tolerant inbred lines across the population for screening. The parental lines used as checks, had relative water contents of 60% for Screen-house. Experiment one and a relative water

content range of 74 and 22% for Screen-house experiment two with the parental checks of 40 and 35% for IT93K-503-1 and IT97K-279-3 respectively. These results are based on the summarized and selected potential tolerant and susceptible lines used for field screening for drought (Tables 2 and 3).

Proportion of survival for screen-house experiments one and two

The proportion of recovery and survival for screen-house experiment one ranged between 93 and 5% for the potential tolerant and susceptible inbred lines with the

Table 2. Screen-house 1 chlorophyll content, relative water content, and proportion of survived seedlings for the potential tolerant and susceptible inbred lines selected for field drought evaluation.

RILS	Block	Chlorophyll content		Relative water content week 2	Proportion of survived seedlings	
		Week 3	Week 4		Untransformed (%)	Arcsine transformed
84	1	40.49	26.48	0.3986	93	1.4296
406	17	32.99	24.13	0.551	92	1.0685
223	17	35.69	29.83	0.591	92	1.0685
75	3	34.29	23.43	0.3818	77	0.9236
186	3	31.99	26.83	0.4218	77	0.9236
353	14	28.89	16.03	0.5947	72	0.8263
398	9	36.34	25.48	0.6625	71	0.8424
20	4	36.24	28.18	0.3986	71	0.92
38	18	35.59	21.28	0.5599	68	0.7603
28	10	38.74	21.08	0.5384	63	0.7274
230	18	39.29	14.48	0.5399	58	0.6284
131	3	34.59	32.93	0.4618	57	0.5792
116	6	33.14	19.28	0.6099	48	0.514
325	4	32.74	17.78	0.5192	44	0.5648
255	7	28.54	12.22	0.5699	42	0.451
57	16	53.04	14.23	0.4851	42	0.4459
189	12	30.24	16.03	0.4759	40	0.4169
142	12	32.14	9.03	0.5659	40	0.4169
408	9	35.74	9.88	0.3925	21	0.2198
55	12	32.24	22.23	0.5059	20	0.2067
78	7	29.94	8.12	0.5199	12	0.1396
396	5	35.69	22.13	0.5333	5	-0.0182
Standards						
IT 93K-503-1		35.68	25.26	0.579	72	0.91
IT 97K-279-3		36.3	23.5	0.575	49	0.55
SED for standards				0.033	7	0.111
SED for RILs in same block				0.1044	33	0.49
SED for RILs in different blocks				0.1442	38	0.565

Table 3. Screen-house 2 chlorophyll content relative water content, and proportion of survived seedlings for the potential tolerant and susceptible inbred lines selected for field drought evaluation.

Genotype (RILs)	Block	Chlorophyll content			Relative water content Oct 14, 2015	Proportion of survived seedlings	
		Week 1	Week 2	Week 4		Untransformed (%)	Arcsine Transformed
84	2	46.72	40.5	20.7	0.2259	100	1.52
325	9	41.43	34.03	21.4	0.6988	100	1.59
230	16	43.48	36.07	18.8	0.6379	97	1.44
406	5	46.76	42.11	31.3	0.7449	95	1.38
223	10	40.71	34.85	19	0.6396	95	1.38
38	11	47.76	39.97	20.1	0.6849	92	1.14
131	8	42.36	35.31	11.7	0.7292	90	1.12
75	12	44.25	38.22	25	0.701	80	0.96
398	16	45.28	39.47	29.7	0.6079	77	0.87
186	19	41.87	32.76	20.3	0.7036	77	0.87
20	2	40.82	35.5	22.8	0.4259	72	0.8
255	7	40.58	32.39	11.5	0.5627	62	0.71

Table 3. Contd.

28	14	45.97	38.25	23.3	0.7157	62	0.73
189	17	47.15	37.17	6.5	0.7339	43	0.47
396	2	41.22	36.9	20.8	0.4259	42	0.43
116	11	38.56	30.97	9.0	0.7649	42	0.43
353	7	42.98	39.79	22.7	0.6727	42	0.48
57	6	42.71	34.33	16.1	0.6797	32	0.37
55	15	46.7	40.03	16.2	0.7449	25	0.18
408	12	45.65	34.32	12.5	0.681	20	0.24
78	6	45.21	35.03	16.6	0.6997	12	0.16
142	8	48.46	35.41	4.8	0.5492	0	0
Standard							
IT 93K-503-1		39.56	38.56	18.78	0.6975	91	1.25
IT 97K-279-3		35.43	33.45	25.85	0.675	80	1.02
SED for standards		0.81		1.54	0.012	4.5	0.095
SED for RILs in same block		2.64		5.01	0.038	14.5	0.309
SED for RILs in different blocks		0.64		6.92	0.053	20.1	0.427

parental checks scores of about 72 and 49% for IT93K-503-1 and IT97K-279-3 respectively. The proportion of survival for screen-house two were relatively higher compared to screen-house one. Survival for some inbred lines were 100%, whereas the lowest scored 54% with the parental checks 91 and 80% for IT93K-503-1 and IT97k-279-3 respectively. Results for the selected potential drought tolerant and susceptible inbred lines for screen-house screening at the seedling stage for experiment one and two are summarized and presented in Tables 2 and 3 respectively.

Mean squares of measured traits for cowpea inbred lines evaluated in the screen -house for tolerance to drought

There were significant differences ($p < 0.05$) for chlorophyll contents for the 200 inbred lines used in the study. Chlorophyll contents taken during 7, 14, 21 and 28 days after water stress treatment significantly varied among the inbred lines, but no significant differences were observed for the parental checks used for the study (Table 4). Significant differences were observed for relative water content taken at 14 days of stress imposition, leaf wilting and recovery.

Pearson correlation coefficient between relative water content, chlorophyll and leaf wilting

Relative water contents at 14 days of water stress correlated negatively with chlorophyll at 7, 14, and 28 days of stress imposition. Relative water content also correlated negatively with leaf wilting at 7 days but

positively correlated with leaf wilting at 14, 21 and 28 days of stress imposition. Relative water content at 28 days of stress however, correlated positively with chlorophyll at 7, 14, 21 and 28 days of stress and leaf wilting at 7 and 28 days but correlated negatively with leaf wilting at 14 and 21 days during the stress imposition (Table 5). Leaf wilting after 7 days of water stress correlated positively with chlorophyll for 7, 14, 21 and 28 days of water stress, however, leaf wilting 14, 21 and 28 days negatively correlated with chlorophyll at 7, 14, 21 and 28 days of stress imposition.

DISCUSSION

Breeding for drought tolerance for cowpea improvement using various techniques, has been exploited by many research scientists all over the world, especially in cowpea producing countries. The use of wooden boxes has been found to be the most appropriate, fast and rapid screening approach for seedling drought tolerance for shoot related traits such as the relative water content, (Aref et al., 2013; Bogale et al., 2011; Pirzad et al., 2011; Pungulani et al, 2013); leaf wilting, chlorophyll contents, (Steidle Neto et al. 2017), and the estimation of proportion of survived seedlings after recovery (Olubunmi, 2015; Muchero et al., 2008; Tomar and Kumar, 2004). This has been the most successful approach for evaluating large populations and subsequent selection for field drought assessment of genotypes (Singh et al., 1999b). In this study, the use of wooden box technique to rapidly screen 200 inbred lines over a four-week period and a repeat for confirmation was helpful to discriminate among inbred lines for tolerance to seedling drought. This corroborates with

Table 4. Analysis of variance for cowpea inbred lines evaluated at the Screen-house for tolerance to seedling stage drought using the rapid screening approach.

Source variation	of df	CHL 7 DAP	CHL 14 DAP	CHL 21 DAP	CHL 28 DAP	RWC 14 DAP	RWC 28 DAP	LW 7 DAP	LW 14 DAP	LW 21 DAP	LW 28 DAP	Recovery
Block	19	6.42ns	8.32ns	5.94ns	24.68ns	0.0047*	0.021	0.438ns	0.870ns	1.33ns	0.36ns	3.25
Families/ inbreds	199	9.51ns	0.0097*	0.00167*	37.08*	0.005*	0.0084*	0.0049*	0.009*	1.741ns	0.54ns	0.005*
Controls	1	4.81ns	0.03ns	15.64ns	1.40ns	0.012*	0.0001ns	0.001*	0.005*	0.33ns	0.33ns	0.008*
Residual	21	5.81	7.18	10.37	22.63ns	0.002	0.019	0.328	0.740	1.34ns	0.45	6.23
Total	239											

df = degree of freedom; CHL= chlorophyll; RWC = relative water content; LW = leaf wilting, ns; = Non-Significant; ** p < 0.01; * P < 0.05 DAP= days after planting.

Table 5. Pearson correlation for chlorophyll, relative water content, and leaf wilting for the inbred lines evaluated under drought stress in the screen-house for seedling tolerance above diagonal (screen-house experiment 1) below diagonal (screen-house experiment 2).

	CHL 7 DAP	CHL 14 DAP	CHL 21 DAP	CHL 28 DAP	RWC 14 DAP	RWC 28 DAP	LW 7 DAP	LW 14 DAP	LW 21 DAP	LW 28 DAP
CHL 7 DAP	-	0.6611*	0.4925*	0.2160*	0.0213	0.0061	-	0.0646	-	-0.119
CHL 14 DAP	0.6949*	-	0.4246*	0.1556*	-0.1504*	-0.098	-	0.0127	-	-0.088
CHL 21 DAP	0.4352*	0.7475*	-	0.3135*	-0.019	0.0448	-	0.0974	-	-0.044
CHL 28 DAP	0.4143*	0.6728*	0.6273*	-	-0.095	0.1694*	-	0.0421	-	-0.055
RWC 14 DAP	0.0209	-0.055	0.1099	-0.042	-	0.1	-	0.0609	-	-0.082
RWC 28 DAP	0.1676*	0.3787*	0.3793*	0.3883*	0.028	-	-	-0.03	-	0.046
LW 7 DAP	-0.006	0.0961	0.0456	0.0722	-0.115	0.0148	-	0.1736*	-	-
LW 14 DAP	0.1222	0.022	0.03	0.0125	0.015	-0.08	0.0548	-	-	-
LW 21 DAP	-0.036	-0.1916*	-0.1966*	-0.123	-0.042	-0.052	0.0617	-0.005	-	-
LW 28 DAP	0.1218	0.0761	0.0617	0.1081	0.1221	-0.085	-0.052	0.1736*	0.0332	-

** p < 0.01; * P < 0.05; . RWC = relative water content, CHL = chlorophyll, LW = leaf wilting, DAP = days after planting.

similar study by Soltys-Khan et al. (2016) who reported that a fast screening tool would be helpful in selecting valuable genotypes with defined growth strategies that translates to drought tolerance and are therefore suitable for breeding experiments since the phenotype is controlled by genes derived from both parents.

In the current study, water stress significantly reduced the chlorophyll contents for both the

parental checks and the inbred lines used. This result corroborates with similar drought related studies report that, drought related traits such as leaf area index, leaf area duration and chlorophyll contents decreased as the water stress duration increased (Khan et al., 2015; Pirzad et al., 2011; Deblonde and Ledent, 2000). Also, Yuan et al. (2016), who in their study on the effects of different levels of water stress on leaf photosynthetic

characteristics and antioxidant enzyme activities of greenhouse tomato reported that water stress decreased stomatal conductance net photosynthetic rate, photosynthetic rate at light saturation, and chlorophyll content in all development stages of tomato resulting in yield reduction.

Water deficit affects the photosynthetic ability of plants by changing the content and components of

chlorophyll, reducing the net CO₂ uptake by leaves, thereby decreasing activities of enzymes in the Calvin cycle (Cornic and Massacci, 1996; Gong et al., 2005; Lawlor and Tezara, 2009). In this current study, as water stress imposition progressed, there were reductions in the chlorophyll contents for all the inbred lines.

Similar study by Tuberosa (2012), on a three-week drought treatment on Katahdin-derived potato cultivars resulted in a decrease in the leaf water content of the cultivars in relative to the control. There was significant variation among the inbred lines for relative water content (RWC) in this study. This observation corroborates with Zegaoui et al. (2017) who reported that two cowpea land races originating from the arid area, maintained a higher RWC over the duration of the drought stress and transpired less than the landrace from the temperate area. Studies by Bogale et al. (2011) and Pirzad et al. (2011) on wheat genotypes and *Matricaria chamomilla* respectively for drought tolerance reported that changes in the relative water content of leaves are considered as a sensitive indicator of drought stress and more useful indicator of plant water balance (Bogale et al., 2011; Clavel et al., 2005). Therefore, the inbred lines with higher relative water content may have a high potential for survival under field drought conditions and subsequently give good yields. Also, relative chlorophyll values for the screen-house experiment one and two in this study gradually reduced as the water stress advanced. This corroborates studies by Bogale et al. (2011) that water deficit has tremendous effects on chlorophyll fluorescence and leaf gas exchange parameters. Thus, photosynthesis rates decreased with decreases in stomatal conductance. Therefore, the relative water content, chlorophyll contents, leaf wilting, survival, and recovery from drought are very good indices for Screen-house selection for seedling drought tolerance of large populations of inbred lines for drought evaluation in the field (Pungulani et al., 2013; Muchero et al., 2008; Singh et al., 1999c).

Conclusion

There were variations with respect to seedling drought tolerance for the 200 inbred lines using the parents as checks in this experiment. The inbred lines that performed well for recovery were 84, 406, 325, 223, 75, 186, 131, 20, 38, 230, 398 and 353, the susceptible ones were, 142, 78, 55, 57, 408, 255, 396, 116, 189, 255, and 28. The inbred lines that maintained a higher relative water content during stress imposition avoided drought and recovered better, were inbred lines 406, 325, 84, 230, 38 and 75, which recorded higher percentages of relative water contents as well as chlorophyll content.

The inbred lines whose proportion of survival ranged between a score of 100- 60% consistently for screen-house 1 and 2 experiments were selected as the potential tolerant lines. Whereas the ones that had poor

survival and recovery after re-watering; whose recovery ranged between 0-40% were identified as potential susceptible inbred lines. The relative water content and chlorophyll for the potential seedling tolerant lines were in the range of 40-70% and 32-53%. The screen-house experiment was repeated to confirm the potential seedling tolerant and susceptible inbred lines that were subsequently selected for the field screening under managed drought conditions. Most of the potential susceptible inbred lines also had lower chlorophyll contents but the relative water content was most often within average (39 and 50%). This could be due to the environmental conditions prevailing at the time of the second experiment thus leading to the high proportions for survived seedlings across all the treatments for all the blocks. The use of physiological traits in scoring for seedling tolerance in this study has facilitated the classification of the inbred lines into drought tolerant and susceptible inbred lines. The genetic variability found for this morphological trait among these inbred lines in the screen-house study, suggest that opportunity exists for selecting superior genotypes under water limited conditions in the field.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of the parental and hybrid lines- Heterosis and other genetic parameters among *Capsicum annum* genotypes and their hybrids

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In most parts of the world pepper has become a popular spice that makes food hot in addition to its much needed nutrients of carotenoids and vitamins. This report has its objective to evaluate the parents and hybrid of three Nigerian pepper genotypes (Shombo, Tatase and Nsukka yellow pepper) for growth and yield; and to estimate their genetic parameters. Conventional breeding by hand pollination was adopted. The mean performance of the parents and hybrids for morpho-agronomic characters were evaluated. Heterosis and inbreeding depression were estimated. The F₁ hybrids showed superiority over the parental genotypes with *Sho x Nsky-rw* having significantly ($P \leq 0.05$) the highest value in most of the traits. In morphological traits, *Sho x Nsky-rw* hybrid had highest values in canopy diameter, number of leaves, number of nodes and number of branches per plant, while in fruit traits, it also had significantly the highest values in the number of fruits, fruit length and weight of fruits per plant. *Sho x Nsky - rw* had positive heterobeltiosis over the better parent in most of the yield and yield component characters. While *Sho x Nsky - rw* had the highest yield, *Tat x Sho* had the highest fruit wall thickness. The combinations of these traits are important to both the farmers and consumers. Having appropriate parents with genetic variability F₁ pepper plants having distinct superiority above the mid and better parents could be developed.

Key words: *Capsicum annum*, chili pepper; heterosis, hybrid, inbreeding depression.

INTRODUCTION

Pepper is a vegetable grown by small, medium and large producers or integrated to agro-business with a considerable socio-economic importance. *Capsicum* genus has been reported to be one of the important vegetables cultivated in Nigeria which is ranked in the third position after onion and tomato in the economic

market (Maga, 2012). Diverse types of *Capsicum* species are cultivated widely in Africa where they are consumed as traditional vegetable or spice. Due to the great range of pepper products and by-products, uses and forms of consumption, it is an essential spice commodity and has formed an integral part of many diets in the world

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Table 1. Crossed in a complete diallelic mating system without reciprocal crosses to obtain F₁ generation.

Correlation	<i>Nsky - rw</i>	<i>Shombo(Sho)</i>	<i>Tatase(Tat)</i>
<i>Nsky - rw</i>	<i>Nsky-rw</i> ^(x)	<i>Nsky-rw</i> x <i>Sho</i>	<i>Nsky-rw</i> x <i>Tat</i>
<i>Shombo(Sho)</i>	<i>Sho</i> x <i>Nsky-rw</i>	<i>Shombo</i> ^(x)	<i>Sho</i> x <i>Tat</i>
<i>Tatase(Tat)</i>	<i>Tat</i> x <i>Nsky-rw</i>	<i>Tat</i> x <i>Sho</i>	<i>Tatase</i> ^(x)

(Bosland and Votava, 2000; Rodrigues et al., 2012). However, due to the absence of dependable statistics and outlined information, it is difficult to measure the real size and importance of pepper market; however, obtaining improved cultivars are very necessary in sustaining the pepper agro-business (Rodrigues et al., 2012).

Capsicum species are usually self-compatible and self-pollinating crop (Raw, 2000; Onus and Pickersgill, 2004). Some percentage of outcrossing is associated with insect pollinators than with wind (Raw, 2000). Genetic advance (GA) under selection refers to the improvement of characters in genotypic value for the new population compared with the base population under one cycle of selection at given selection intensity (Singh, 2001). Therefore, genetic advance (GA) is important to predicting the expected genetic gain from one cycle of selection. Heterosis is one of the most important genetic tools for the manifestation of different cross combinations in crops and determining their potential for commercial exploitation under different environmental conditions (Ashfaq et al., 2013). This breeding method has been used extensively in agriculture to increase yield and to enlarge adaptability of hybrid varieties in a number of crop species (Meyer et al., 2004). The percentage increase or decrease in traits among the hybrids over the mid-parent and better parents' performance is used to assess the overall performance of these hybrids (Inamullah et al., 2006). Heterobeltiosis expresses the betterment over the better parent; likewise, standard heterosis expresses the heterosis over the standard varieties (Shrestha et al., 2011). The major goal of the research was to improve the red fruited pepper genotypes in relation to aroma and β -carotene; however, this report is aimed at evaluating the hybrid field performance as compared to the parents (*Shombo*, *Tatase* and *Nsukka* yellow pepper) and the estimation of heterosis

MATERIALS AND METHODS

The experiment was conducted at the Botanical Garden, University of Nigeria, Nsukka. The seed sources of the parental accessions were from a three year evaluation programme at the Faculty of Agriculture Farms, University of Nigeria, Nsukka. The F₁ hybrids were generated between the highly aromatic genotype, *Nsukka* Yellow Pepper (*Nsky - rw*) and some red and fat fruited genotypes (*Shombo* and *Tatase*) with a view to improving the red fruited genotypes in relation to aroma from the *Nsky - rw* parent and

improving the carotenoid content of the aromatic yellow pepper genotype (*Nsky - rw*) from the red fruited genotypes (*Shombo* and *Tatase*). Hybridization was conventionally done by hand pollination (Morakinyo and Falusi, 1992) (Table 1).

Seeds from successful crosses were used to generate the F₁ plants. The F₁s were evaluated in the subsequent year and the seeds from F₁ plants were used to raise the F₂ plants. The parents, F₁ and F₂ plants were evaluated in the field using randomized complete design (RCBD). Cultural practices were maintained manually. The weeds were removed by manual weeding with small hoe. The data on plant height, mainstem length, mainstem internode length and canopy diameter were metrically measured in centimeters (cm). The parameter on the number of branches per plant, number of leaves per plant, number of nodes per branch, number of nodes per plant, number of nodes on main stem were collected by numerical count at one hundred to one hundred and five days after planting (DAP). The leaf area (cm²) measurement was taken using the autoamated leaf area meter in the Physiology Laboratory of the Department of Crop Science, University of Nigeria, Nsukka.

The fruit attributes and yield were also studied and data were collected on fruit diameter (cm), fruit length (cm), fruit stalk length (cm) and Pericarp thickness (mm) - measured with Venier Caliper. The number of fruits per plant was by numerical count. Fresh fruit weight (g) per plant was also determined. The fruits were processed and the seeds were sun dried for five days in the Botanical garden; the average of the numerical count of seeds was recorded as number of seeds per fruit. Seed weight / fruit (g) was also determined. The yield in tonnes per hectare was estimated from fruit yield per plant using plant population per hectare.

Statistical and genetic analysis of data

Data collected on the quantitative characters were subjected to analysis of variance (using GenStat Discovery Edition 4 software) to test for significant differences among the treatments, while significant means were separated using Fisher's Least Significant Difference (F-LSD) at $p < 0.05$.

Estimation of Heterosis/ Hybrid vigor was achieved using the formula:

$$\text{Heterosis over the mid parent} = \frac{\bar{F} - \bar{MP}}{\bar{MP}} \times \frac{100}{1}$$

$$\text{Heterosis over the better parent} = \frac{\bar{F} - \bar{BP}}{\bar{BP}} \times \frac{100}{1}$$

$$\bar{F}_1 = \text{Mean of } F_1$$

$$\bar{MP} = \text{Mid parent value}$$

$$\bar{BP} = \text{Better parent value}$$

Differences between selfed (F₂) and outcrossed (F₁) progeny were

Table 2. Mean performance of parents, F₁ and F₂ populations in morphological and yield component characters.

Category		Canopy spread	No. of leaves/plt	Leaf area	Mainstem length	Mainstem internode length	No. of nodes on mean stem	No. of nodes/branch	No. of nodes/plt	No. of branches/plt	Plant height
Parent											
<i>Shombo</i>		52.15	241.1	14.22	22.53	1.9	18.50	7.96	392.43	215.18	61.6
<i>Tatase</i>		37.9	64.27	14.11	16.81	1.5	14.33	8.43	83.87	55.65	49.03
<i>Nsky-rw</i>		51.69	127.33	22.96	17.84	1.31	20.82	7.55	265.94	123.97	43.63
Hybrid											
<i>Sho x Nsky-rw</i>	F ₁	66.92	337.43	24.77	22.78	0.55	27.33	13.54	608.63	233.33	51.71
	F ₂	61.83	144.33	22.13	14.42	0.96	19.00	8.08	216.79	172.85	57.51
<i>Tat x Nsky-rw</i>	F ₁	51.69	116.77	23.06	32.33	1.3	21.67	9.21	187.27	188.24	66.11
	F ₂	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FLSD. 05 (genotype)		9.46	26.54	3.9	1.25	0.26	3.8	2.19	39.05	13.3	9.5

NS = Non-survived.

estimated according to Hedrick and Kalinowski, (2000) using

$$D = 1 - w_s / w_o,$$

D = Inbreeding effect.
 w_s = F₂ value
 w_o = F₁ value

RESULTS AND DISCUSSION

The results of the field evaluation of the parents and hybrids are presented in Tables 2 and 3. There were significant variations across the traits among the parents and hybrids. The values for canopy diameter ranged from 37.9- 66.92 cm. Wider canopies could confer advantage in exposing the leaves to sunlight and more production of the fruit. Equally, high number of leaves together with high leaf area among the F₁ plants of *Sho x Nsky – rw* is advantageous in attracting more sunlight energy thereby increasing

assimilates from photosynthesis. *Shombo* had significantly the highest value in main stem length across the parent and other hybrid. Its hybrid (*Sho x Nsky – rw*) equally significantly had the tallest plants with a mean value of 66.11 cm. This value did not, however, vary from *Shombo* parent and F₂ plants of *Sho x Nsky – rw*. Tall plants could be disadvantageous in mechanical harvesting but could be preferred in positioning the leaves and tender branches for wider contact with sunlight. *Sho x Nsky – rw* F₁ plants had significantly the shortest internodes (0.55 cm). These same F₁ plants (*Sho x Nsky – rw*) also had significantly the highest number of nodes on the main stem, number of nodes/ branch, number of nodes/ plt and number of branches/ plt. This seems to suggest that the inheritance of shorter internodes could result in a significant increase in yield component characters that may have direct or indirect effect on fruit yield. These yield components have been shown to contribute to

yield by having high correlations with fruit yield (Nandadevi and Hosamani, 2003).

The results of the fruit characters showed that *Tatase* had significantly the fattest fruits (2.03 cm diameter) while *Shombo* had significantly the longest fruits (7.76 cm) across the parents and hybrids (Table 3). *Tatase* parent which had the fattest fruits also had the longest fruit stalk length and the highest number of seeds/ fruits. This fat fruited genotype had the highest single fruit weight; however, it sets few fruits which is evidenced by the significantly low number of fruits per plant (14.83). Equally the F₁ hybrids (*Tat x Nsky-rw*) inherited this attribute of low number of fruits per plant (9.33). *Tatase* genotype has its preference in having high single fruit weight and thick pericarp as recorded in fruit wall thickness (3.54 cm) (Table 3). The highly aromatic genotype (*Nsky-rw*) had a relatively high yield (6.64 t/ha) which was significantly higher than the *Tatase* parent and the F₁ hybrids of *Tat x Nsky-rw*.

Table 3. Mean performance of parents, F₁ and F₂ populations in fruit characters.

Category		Fruit diameter	Fruit length	Fruit stalk length	No. of seeds / fruit	Seed wt/ fruit	Single fruit wt	No. of fruits/ plt	Fruit wt/ plt	Fruit yield (t/ha)	Fruit wall thickness
Parents											
<i>Shombo</i>		0.71	7.76	3.85	133.9	0.92	4.26	77.1	333.08	11.1	1.94
<i>Tatase</i>		2.03	3.51	3.95	188.97	0.81	6.65	14.83	97.86	3.26	3.54
<i>Nsky-rw</i>		1.19	3.53	3.24	50.5	0.22	2.87	69.72	199.38	6.64	1.67
Hybrid											
<i>Sho x Nsky-rw</i>	F ₁	0.8	5.9	3.32	39.39	0.33	2.16	156.84	338.47	11.28	1.07
	F ₂	0.86	4.77	3.32	43.73	0.20	2.75	56.87	128.33	1.56	1.64
<i>Tat x Nsky-rw</i>	F ₁	1.24	4.21	3.85	50.37	0.18	3.19	9.33	29.76	1.00	2.20
	F ₂	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FLSD. 05 (genotype)		0.23	0.37	0.4	25.62	0.17	0.68	15.76	48.94	2.3	0.4

NS = Non survived.

The higher numbers of fruits recorded in *Shombo* parent and its F₁ hybrids (*Sho x Nsky - rw*) were translated to higher cumulative yield of 11.1 and 11.28 t/ha, respectively. The red fruited genotype *Shombo* could be used as one of the parents in breeding programs due to its high yield. Shukri et al (2015) reported that genetic distance between parents used in breeding program is required in developing new parents with high performance in line with the breeders' objectives. It had earlier been reported that *Shombo* has a *frutescens* origin while Nsukka yellow pepper (*Nsky - rw*) is purely *Capsicum annum* (Morakinyo and Falusi, 1992). This tends to point out the genetic distance between these two parents, thus favouring the observed high performance.

Canopy diameter, number of leaves/ plt, number of nodes/ plt, leaf area and number of branches/ plt showed positive heterosis over the mid-parents in both hybrids, and all except number of leaves/ plt in *Tat x Nsky-rw*, showed positive

heterobeltiosis over the better parent (Table 4). These traits are the major traits that contribute to yield in peppers. Nandadevi and Hosamani, (2003) reported a high correlation between these traits and pepper yield. It, therefore, implies that the increase in these yield component traits could significantly improve fruit yield among the F₁ plants. The F₁ hybrids of *Tat x Nsky-rw* had positive heterosis over the mid and better - parents (+42.69 and +34.84, respectively) in plant height. This peculiarity puts these F₁ plants at advantage in hand harvesting since taller plants are preferred. All the major yield component traits showed inbreeding depression in F₂ among *Sho x Nsky - rw* plants. This is expected as there are allelic segregations at F₂ and pepper being predominantly self - pollinating would have high heterozygosity at F₁. High heterozygosity favours heterosis in many plant species. The F₂ seeds of *Tat x Nsky-rw* failed in germination; this phenomenon had previously been observed in

crosses by some researchers (Silva et al., 2017).

The estimation of heterosis over the mid and better parent and inbreeding depression in F₂ for fruit characters is presented in Table 5. The number of fruits/ plt is a major trait contributing to fruit yield in peppers and it has positive heterosis over both the mid and better - parent in *Sho x Nsky - rw* (+113.65 and +103.42, respectively). It has been reported that number of fruits/ plt could predict yield with a high prediction accuracy (Abu et al., 2015).

Fruit weight together with fruit yield also had a positive heterosis over both the average and better parental values in *Sho x Nsky - rw*. Superiority over the better parental values (heterobeltiosis) has been reported to be more effective than heterosis, particularly in the breeding of self-pollinating crops (Shukri et al., 2015). It should also be noted that most of the fruit characters had negative heterosis in *Tat x Nsky-rw*. *Tatase* usually sets few fat fruits with high

Table 4. Estimation of heterosis over the mid and better parents and inbreeding depression for morphological traits.

Character	Heterosis % <i>Sho x Nsky-rw</i>		Heterosis % <i>Tat x Nsky-rw</i>		Inbreeding Depression <i>Sho x Nsky-rw</i>	Inbreeding Depression <i>Tat x Nsky-rw</i>
	\overline{MP}	\overline{BP}	\overline{MP}	\overline{BP}		
	Canopy diameter	+28.89	+28.32	+15.38		
No. of leaves/plt	+83.17	+39.95	+21.89	-8.29	0.57	-
Leaf area	+33.24	+7.93	+24.38	+0.44	0.11	-
Mainstem length	+12.83	+1.11	+86.56	+ 81.22	0.37	-
Mainstem internode length	-65.84	-71.05	-7.80	-13.33	-0.75	-
No. of nodes on mainstem	+39.01	+31.27	+23.27	+4.08	0.30	-
No. of nodes/branch	+74.48	+70.10	+15.27	+9.25	0.40	-
No. of nodes/plt	+84.89	+55.09	+7.07	+29.58	0.64	-
No. of branches/plt	+37.59	+8.44	+10.60	+51.84	0.26	-
Plant height	-1.73	-16.06	+42.69	+34.84	-0.11	-

Table 5. Estimation of heterosis over the mid and better parents and inbreeding depression for fruit characters.

Character	Heterosis % <i>Sho x Nsky-rw</i>		Heterosis % <i>Tat x Nsky-rw</i>		Inbreeding Depression <i>Sho x Nsky-rw</i>	Inbreeding Depression <i>Tat x Nsky-rw</i>
	\overline{MP}	\overline{BP}	\overline{MP}	\overline{BP}		
	Fruit diameter	-15.79	-32.77	-22.98		
Fruit length	+2.61	-25.88	+19.60	+19.26	0.19	-
Fruit stalk length	-6.48	-13.77	+6.94	-2.53	0.00	-
No. of seeds/fruit	-57.27	-70.58	-57.93	-73.34	-0.11	-
Seed wt/fruit	-42.1	-64.13	-65.39	-77.78	0.39	-
Single fruit wt	-32.71	-49.30	-33.26	-52.03	-0.27	-
No. of fruits/plt	+113.65	+103.42	-77.93	-86.62	0.64	-
Fruit wt/plt	+27.13	+1.62	-79.98	-85.07	0.62	-
Fruit yield t/ha	+27.17	+1.62	-79.80	-84.94	0.86	-
Fruit wall thickness	-40.88	-44.85	-15.71	-37.85	-0.53	-

single weight value. Inbreeding depression was positive among the F₂ plants of *Sho x Nsky-rw* while in *Tat x Nsky-rw*, there were no survivals.

González et al. (2014) associated inbreeding depression with embryo mortality and a reduction in plant vigor. This could explain the germination

failure of the F₁ seeds of *Tat x Nsky-rw* hybrids and the reduction in vigor among the F₂ hybrids of *Sho x Nsky-rw*. In conclusion, the very short

internode length observed in *Sho* x *Nsky* – *rw* hybrid was advantageous as it had the highest values in major morphological traits with high weightage to yield and in fruit yield. Equally, while *Sho* x *Nsky* – *rw* had the highest yield, *Tat* x *Sho* had the highest fruit wall thickness. The combinations of these traits are important to both the farmers and consumers. The genetic distance between the *Shombo* and *Nsukka* yellow pepper was advantageous in raising F₁ hybrids with high performance above the better parent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Genetic action and potence ratio of maize in an 8×8 diallel cross growing under saline condition

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Twenty-eight hybrids of *Zea mays* L. produced in 2015-16 from 8×8 half diallel mating design were evaluated along with their parents in 2016-2017 under saline condition to find out genetic action and potency ratio of some agronomic and yield contributing traits. Genetic action and potency ratio were analyzed following Hayman's diallel analyses and Smith's formula, respectively. Genetic parameter like additive variance (D), dominance variance (H1), proportion of positive and negative genes in the parents (H2), relative frequency of dominant and recessive alleles in the parents (F), dominance effect over all loci in heterozygous phase (h2) suggested that traits like days to tasseling, days to silking, anthesis silking interval, ear height and number of grains/plant are governed by dominant alleles in these traits. Contrariwise, the data of plant height and 100-grain weight were shown to have higher frequency of recessive alleles. Proportion of genes with positive and negative $[(4DH1)0.5 + F]/[(4DH1)0.5 - F]$ effects suggested the asymmetrical distribution of dominant and recessive alleles for all the traits except yield/plant. Under saline condition heritability in narrow sense (h²_n) was found very low which indicates a possible strong influence of stress in the growing environment. These parameters along with Vr-Wr graphs and potency ratio indicated overdominance in desirable direction, and thus, heterosis breeding is important to improve those traits in maize under salinity.

Key words: Maize, salinity, genetic parameter, Vr-Wr, potence, inheritance.

INTRODUCTION

Maize (*Zea mays*) is the solely cultivated species of the genus 'Zea' and the tribe Maydeae. It is the oldest crop species domesticated as food crop in the world which is a C₄ crop. Being a C₄ crop, maize possesses most potentiality of ensuring food security in the coming days (Chohan, 2012). It can be cultivable in broad climatic

conditions worldwide. Globally, maize occupies the third most important position as a crop. Maize is compatible with wide range of agro-climatic zones. The suitability of maize to diverse environments is unparalleled to any other crops (Hossain et al., 2016). Maize can be grown from below sea level to higher altitudes like >3000 m.

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The rainfall range is around 250 mm to greater than 5000 mm per year. The life cycle of maize can vary from 3 to 10 months (Sheikh et al., 2017). According to FAO (2016), in the year 2016 total maize cultivation area was 188 million hectare (ha) while production was 1050.1 million ton and average yield of 5.64 ton ha⁻¹.

Moreover, the multipurpose uses of maize coupled with its maximum grain yield make it popular globally. It is used as food for human, feed for poultry, livestock and fish. In Bangladesh, poultry and fish feed industry is increasing. In parallel with this, maize grain demand is also increasing in many folds (Ali et al., 2009). Therefore, high yielding maize hybrid developing program has been executed worldwide. To achieve yield improvement through genetic improvement approaches, gene action determination is essential for conveying necessary breeding strategies. By understanding the nature of gene action, governing a specific trait is essential for improving that particular character which will ultimately increase the yield any crop. Additionally, the choice of a fit breeding program depends on the understanding of the nature of gene action of yield and yield related traits. On one hand, hybrid development program prefers dominance gene action, whereas on the other hand, additive gene action can efficiently improve any character (Hossain et al., 2016). Diallel analysis system was extensively used in heredity related research to investigate the inheritance strength of any important trait in a set of genotypes (Yan and Hunt, 2002). Components of genetic control can assist breeders to select competent parents for a crossing program. Thus, gene action study facilitates decision making of a suitable breeding procedure for genetic improvement of various quantitative traits (Jinks and Hayman, 1963; Walters and Morton, 1978; Reza et al., 2004; Begum et al., 2018).

Information about inheritance pattern of any specific trait can be obtained from Hayman numerical approach (Hayman, 1954a, b) while Griffing (1956) provides a feature on genetic action of parental lines. Diallel crosses have been used for a long time in genetic research to determine the inheritance of a trait among a set of genotypes and to identify superior parents for hybrid or cultivar development (Aliu et al., 2009). These methods have been highly practiced in different crops like maize (Njeri et al., 2017; Owusu et al., 2017; Lay and Razdan, 2017; Brahmhatt et al., 2018), rice (Huang et al., 2015; Kundan et al., 2013), Brassica (Tian et al., 2017) and cassava (Tumuhimbise et al., 2014). Hence, it is necessary to understand the nature and magnitude of gene action as well as combining ability of yield and its attributes.

Besides knowing the genetics of the germplasm, it also becomes a crucial problem to breed for problematic areas of the world. Salinity is a growing threat for crop production and growth throughout the world. In arid and semi-arid regions, salinity level increases due to water shortage and elevated temperature. In Bangladesh,

salinity affected area is increasing every year. In Bangladesh, during 1973, salinity affected 83.3 million hectares of land; this was increased to 102 million hectares by the year 2000. After that, salinity affected a recorded 105.6 million hectares during 2009 (SRDI, 2010). Over the last 35 years, salinity has increased around 26% in the coastal region of Bangladesh (Mahmuduzzaman et al., 2014). Therefore, saline tolerant maize variety development is a time demanding research in Bangladesh.

The present investigation of 8×8 diallel cross maize without reciprocal crosses was undertaken to supplement genetic parameters interpretations, pinpoint which parents contain the preponderance of dominance/recessive genes with increasing/decreasing character attributes, and isolate superior inbred lines and better combining parents for utilizing their salinity affected area of Bangladesh in future breeding programs. Considering those, we analyzed the genetic action of maize in an 8×8 diallel progeny growing in saline soil to understand the nature of gene action in saline condition for developing suitable hybrids for saline area.

MATERIALS AND METHODS

Plant materials

Eight maize inbred lines (CZ-28, CZ-29, BIL-65, CZ-36, CZ-12, CZ-26, CZ-24 and 9MG) collected from International Maize and Wheat Improvement Center (CIMMYT) and Plant Breeding Division, Bangladesh Agricultural Research Institute (BARI) were crossed in a diallel fashion excluding the reciprocals during the rabi season (winter) in 2015-2016 at BARI, Gazipur, Bangladesh. The resulting 28 F₁'s and their 8 parents were evaluated in a randomized complete block design (RCBD) with three replications in saline area of Agricultural Research Station, Benarpota, Satkhira (22.43° N latitude and 89.06° E longitude), Bangladesh in the subsequent (winter) season of 2016-2017. The soil salinity level was also monitored in the growing location by a digital EC meter (Hanna 993310). It was found that salinity level increased throughout the plant growth period which ranged from 3.3 to 9.2 dS/m (Figure 1). This increasing tendency of salinity is due to depletion of soil moisture, because there was no rain in this period.

Experiment settings, crop management and data recording

Seeds of each entry were sown in two rows of 4 m plot. The spacing between rows was 60 cm and plant to plant distance was 25 cm. Fertilizers were applied at 250, 55, 110, 40, 5 and 1.5 kg ha⁻¹ of N, P, K, S, Zn and B, respectively. One plant per hill was maintained after proper thinning. Agronomic and yield related traits of the plants were recorded on five randomly selected competitive plants. Agronomic trait includes days totasseling, days to silking, anthesis silking interval (days), plant height (cm) and ear height (cm). In each plots when 50% plants produced male flower, it was recorded as days to tasseling. Similarly, for days to silking, 50% silking of the plot was recorded. For the trait anthesis silking interval (ASI), days between 50% anthesis and 50% silking was considered. For plant height, ear height as well as yield related recorded traits like number of grains/plants, 100-grain weight (g) and grain yield (g) were measured from randomly selected 10

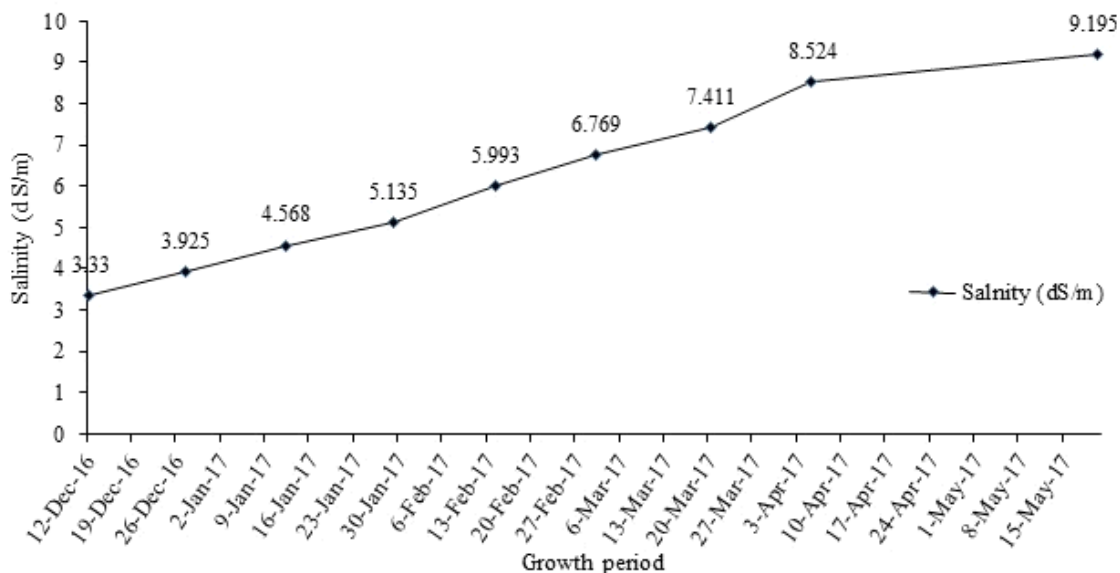


Figure 1. Salinity level of the location during the growing period of maize.

Table 1. Analysis of variance for the 8 × 8 half diallel population of maize.

Source variation	of	df	DT	DS	ASI	EH (cm)	PH (cm)	NG/plant	100-GW(g)	Yield/plant (g)
Replication		2	12.8*	11**	18.4*	2729.3**	2718**	167	14.02**	2.03
Genotype		35	72.5**	112.7**	30.4**	431.6**	1108**	6221**	63.04**	9283.8**
Parent(P)		7	29.5**	34.1**	5.5	329.8**	766*	433*	5.71	91.9*
Offspring(f)		27	32**	13**	24**	332**	659*	4407**	52.60**	1185.9**
Pvsf		1	1466**	3348**	377**	3828.3**	15619**	95899**	746.35**	292270**
Error		70	2.96	2.1	3.8	91.2	351	213	4.80	37.55

DT=days to tasseling, DS=days to silking, ASI=anthesis-silking interval (days), PH=plant height (cm), EH=ear height (cm), NG/Plant=number of grains/plant and 100-GW=100-grain weight (g).

plants for each replication.

Statistical analysis

The statistical analysis of variance was performed as described in Sharma (1988). The genetic parameters of Hayman's graphical and numerical approach (1954a, b) like additive variance (D), dominance variance (H₁), proportion of positive and negative genes in the parents (H₂), relative frequency of dominant and recessive alleles in the parents (F), dominance effect over all loci in heterozygous phase (h₂), environmental variance (E), along with their allied parameters, mean degree of dominance (H₁/D)^{0.5}, proportion of genes with positive and negative [(4DH₁)^{0.5} + F]/[(4DH₁)^{0.5} - F] effects in the parents and heritability in narrow sense (h²_n) was calculated as described in Sharma (1988). Vr-Wr graphs were plotted in MS Excel according to Singh and Chaudhary (1985).

Potency ratio was calculated according to Smith (1952) to determine the degree of dominance as follows: $P = F_1 - M.P. / \{0.5(P_2 - P_1)\}$. Where, P: relative potency of gene set, F₁: first generation mean, P₁: the mean of lower parent, P₂: the mean of higher

parent, M.P.: mid-parents value = (P₁ + P₂)/2.

RESULTS AND DISCUSSION

Analysis of variance

The analysis of variance showed that mean squares for the parent and diallel hybrid lines were significant at P ≤ 0.05 for the traits anthesis silking interval and days to tasseling while the values of other traits were highly significant (P ≤ 0.01) (Table 1). Similarly, the mean sum of squares of genotypes were highly significant for all the recorded eight traits. In case of parents, all the traits, except anthesis silking interval and 100-grain weight, were significant. On the other hand, the values of offspring and parent vs offspring were highly significant. The significant variation among the values of the traits signified the existence of divergence and genetic

Table 2. Genetic variance components and related statistics for 8 traits in an 8×8 diallel cross (without reciprocal cross) of maize.

Genetic parameter	DT	DS	ASI	EH (cm)	PH (cm)	NG/plant	100-GW(g)	Yield/plant (g)
D	8.76**	10.58**	0.42**	55.09**	84.18**	63.94**	0.21**	18.48**
F	10.55**	16.27**	0.63**	74.20**	-10.76**	163.76**	-2.14**	-1.15**
H ₁	79.19**	114.87**	35.64**	442.41**	799.72**	7921.27**	71.87**	1632.99**
H ₂	-1819.1**	-2299.83**	-254.7**	-1415.8**	-64398.97**	-12416.05**	-2260.70**	-7690.94**
h ²	2688.4**	2773.44**	0.09**	1248.16**	5982.57**	3482.35**	174.74**	94.14**
E	1.08**	0.78**	1.42**	54.84**	171.28**	70.69**	1.69**	12.19**
(H ₁ /D) ^{0.5}	3.01	3.29	9.18	2.83	3.08	11.13	18.28	9.40
H ₂ /4H ₁	-57.17	-49.95	-1.79	-7.99	-20.13	-3.92	-7.86	-1.18
[(4DH ₁) ^{0.5} +F]/[(4DH ₁) ^{0.5} -F]	1.50	1.61	1.1779	1.62	0.96	1.260	0.572	0.993
h ² n (%)	1.07	9.0	1.03	9.0	5.0	0.80	0.30	1.1

*Significant at 5% level, ** Significant at 1% level; Additive variance (D), Dominance variance (H₁), proportion of positive and negative genes in the parents (H₂), Relative frequency of dominant and recessive alleles in the parents (F), Dominance effect over all loci in heterozygous phase (h₂), Environmental variance (E), Mean degree of dominance (H₁/D)^{0.5}, Proportion of genes with positive and negative effects in the parents [(4DH₁)^{0.5} + F]/[(4DH₁)^{0.5} - F], heritability percentage (narrow sense (h²n)), DT=days to tasseling, DS=days to silking, ASI=anthesis-silking interval (days), PH=plant height (cm), EH=ear height (cm), NG/Plant=number of grains/plant and 100-GW=100-grain weight (g).

variation among individuals (Chohan et al., 2012). Importantly, the highly significant mean sum of squares at 1 degree of freedom (df) indicated the possibility of heterosis for the traits.

Genetic parameter of different traits in maize under salinity

Gene action was clarified by genetical analysis of Hayman (1954a, b). The analysis of variance components indicated that both additive (D) and dominance variance (H₁ and h²) are all significant for the 8 studied traits (Table 2). This result indicated the possibility of the traits are conditioned by both additive and dominance gene action. However, dominance components are more predominant than additive component. The dominance is also reflected by overall dominance effect over all loci in heterozygous phase (h²). Importantly, higher values of H₂ than H₁ with negative sign indicated the unbalanced distribution of dominant and recessive alleles with negative effects in the parents under salinity stress (Table 2). The size of mean degree of dominance (H₁/D)^{0.5} was categorized as (H₁/D)^{0.5} = 0, mean no dominance, (H₁/D)^{0.5} = 1, mean complete dominance, (H₁/D)^{0.5} > 1, mean over dominance and (H₁/D)^{0.5} < 1 mean partial dominance. The study showed that under salinity condition, the values of (H₁/D)^{0.5} were > 1 for all the traits suggesting prevailing over dominance in all the loci for all the traits. The environmental component (E) was significant and in case of ASI, it was higher than D and H₁ (Table 2) noticing higher environmental effect in expressing the trait. The proportion of dominant (p) and recessive (q) alleles is ascertained by the ration [(4DH₁)^{0.5} + F]/[(4DH₁)^{0.5} - F]. Its value explains that [(4DH₁)^{0.5} + F]/[(4DH₁)^{0.5} - F] ≅ 1.0 means nearly equal proportion of dominance and

recessive alleles in parents, that is, symmetrical distribution; p=q=0.5. If its value is >1.0, it refers to an excess of dominant alleles and the minority of recessive alleles (p>q) while <1.0 means minority of dominant alleles and excess of recessive alleles (p<q). In the present study, this ration was >1.0 for days to tasseling, days to silking, anthesis silking interval, ear height and number of grain/plant (Table 2) indicating of excess of dominant alleles in these traits. Contrariwise, the data of plant height and 100-grain weight exhibited higher frequency of recessive alleles. However, yield/plant had almost symmetrical distribution of the alleles.

Another important genetic parameter, mean covariance of additive and dominant variance expressed by F is presented in Table 2. The value of F expressed that if F=0, it means balanced distribution (p=q=0.5); F>0 (+) means dominant alleles are more frequent than recessive alleles (p>q); F>0 (-) means recessives are more prevalent than dominant alleles (p<q). The value of F (>0) for days to tasseling, days to silking, anthesis silking interval, ear height and number of grain/plant also confirmed excess of dominant alleles. The proportion of dominant genes with positive or negative effects in parents is determined by the ratio: H₂/4H₁ with the maximum theoretical value of 0.25, which stands up when p=q=0.5 in all loci. A deviation from 0.25 would be irregular when p≠q. The values of H₂/4H₁ deviated for all the traits, thus, dominance genes having increasing and decreasing effects on all the traits are irregularly distributed in the parents. These findings confirmed our previous study (Begum et al., 2018). However, the lower heritability estimate (h²n) predicted that the traits were very much influenced by environmental effect. Present results are in good agreement with Hussain et al. (2014) and Irshad-ul-Haq et al. (2010) where they found over dominance for pollen shedding and anthesis -silking interval under drought condition.

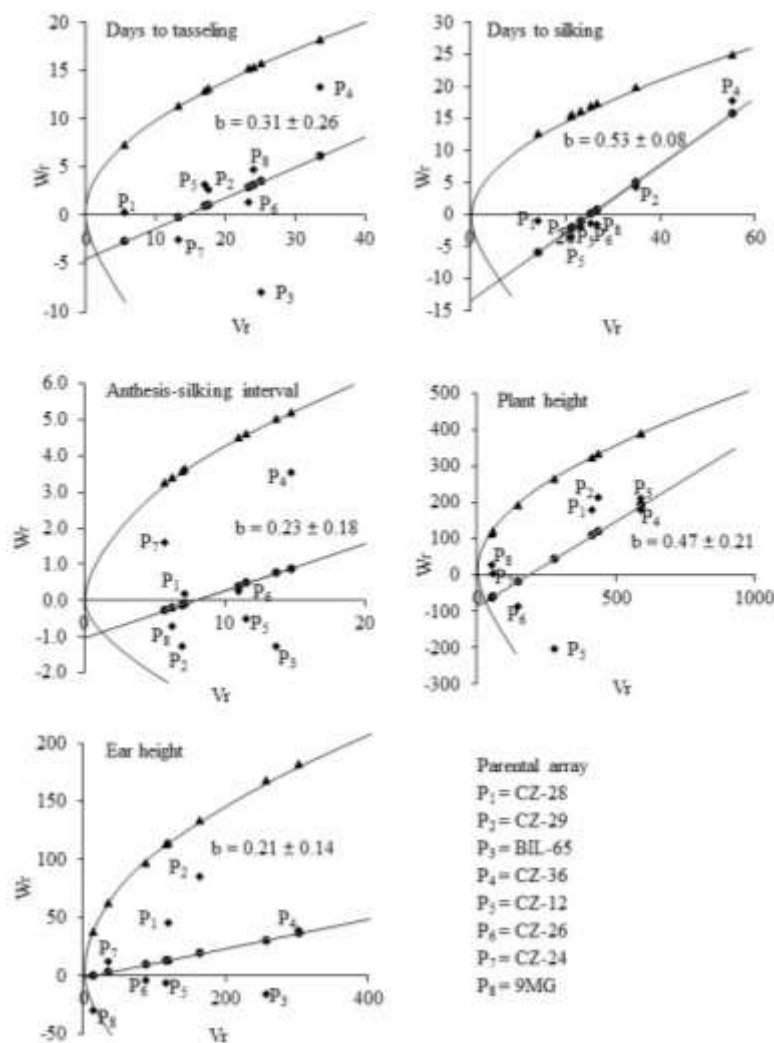


Figure 2. Vr-Wr graphs for days to tasseling, days to silking, anthesis-silking interval, plant height and ear height.

Vr-Wr graph

Hayman's graphical approach to diallel analysis is based on monogenic additive model (Figures 2 and 3). This approach helps to understand the genetic action of the parental lines involved in a diallel mating system which is reported in many crops (EL-Hosary, 2014; Begum et al., 2018; Iftekharruddaula et al., 2008; Boye-Goni and Marcarian, 1985; Rohman et al., 2006). Vr-Wr graphs are the two dimensional depiction made based on the parental variance (V_r) and parent offspring co-variance (W_r). The average level of degree of dominance could be understood by the nature (+/-) and magnitude of 'a' (the Y intercept). The regression line passed above the point of origin suggesting partial dominance for controlling the trait. The regression line intersected W_r axis above the point of origin suggesting partial dominance for controlling the trait. The regression line intersected below

the point of origin suggesting over dominance for controlling the trait. The distribution of array points indicated parental order of dominance which is provided by (W_r+V_r) values. For any trait, the parents containing dominant alleles will fall closer to the point of origin and showed lower value of (W_r+V_r) while, parent with maximum frequency of recessive alleles will fall far from the origin.

The W_r/V_r graphs of days to tasseling, days to silking, anthesis-silking interval, ear height and plant height are presented in Figure 2. In most of the cases, the regression line incepted below the origin suggesting presence of overdominance controlling the traits which was also depicted by $(H1/D)^{0.5}$. Overdominance in different traits in maize was also reported in water stress (Hussain et al., 2014). CZ-36 exhibited maximum frequency of recessive alleles for days to tasseling, silking, anthesis-silking interval, and ear height being

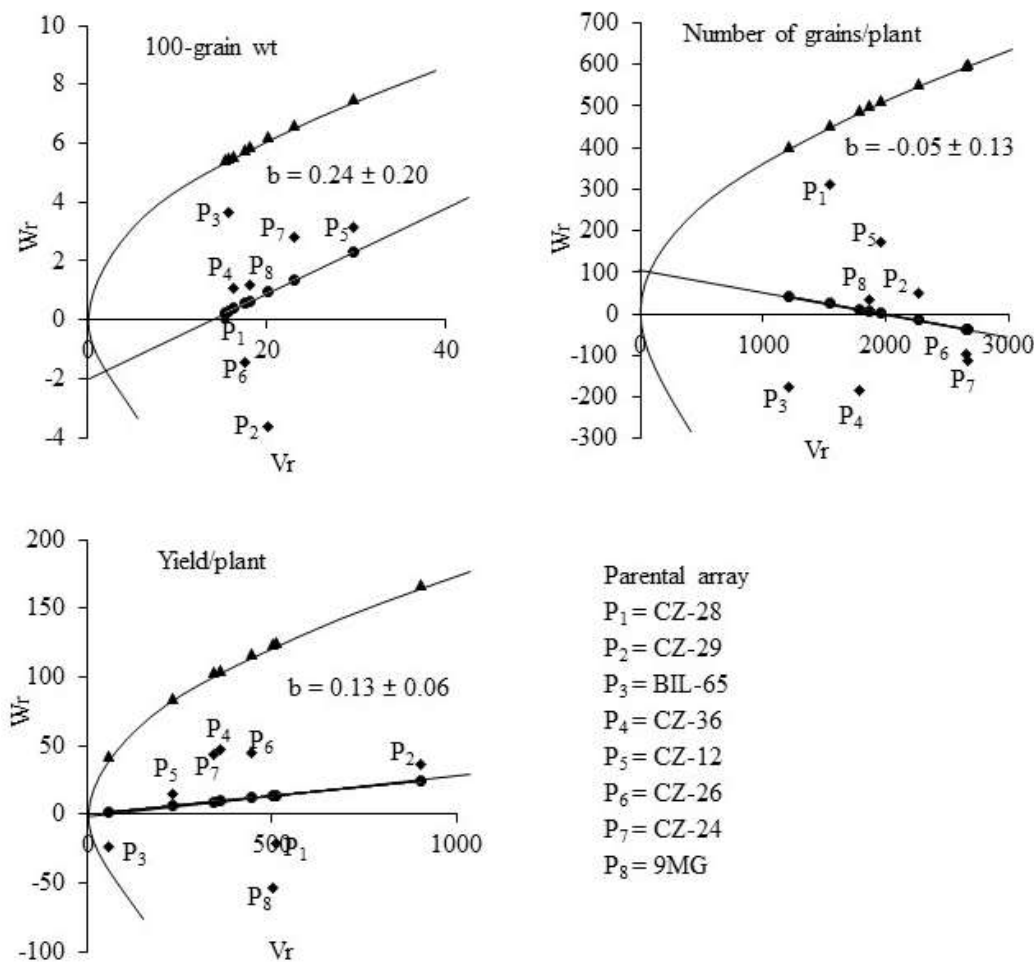


Figure 3. Vr-Wr graphs for 100-grain weight, number of grains/plant and yield/plant.

farthest from the origin while CZ-28 had maximum frequency of dominant alleles for days to tasseling and silking (Figure 2). On the other hand, both BIL-65 and CZ-36 seemed to possess more recessive alleles for plant height and ear height. The parents 9MG, CZ-24, CZ-28 and CZ-29 had more frequency of dominant alleles for anthesis-silking interval while 9MG, CZ-24, CZ-12 and CZ-26 for plant height, 9MG, CZ-24 and CZ-26 for ear height (Figure 2). However, this type of study under salinity is very limited. Hussain et al. (2014) and Irshad-ul-Haq et al. (2010) reported similar results under drought condition.

For yield and related traits, Vr-Wr graphs are presented in Figure 3. The regression lines of 100-grain weights and yield/plant passed below the origin suggesting overdominance action for those traits. On the other hand, the regression line of number of grains/plant passed above the origin and partial dominance effect was present for this trait. The distribution of parents showed most of them possess more dominant alleles for 100-grain weight while CZ-12 had more recessive alleles. For

number of grains/plant, maximum parents had higher recessive allele frequency. On the other hand, parent CZ-29 exhibited maximum alleles frequency for yield/plant due to being farthest from origin while BIL-65 had maximum dominant alleles. Importantly, other parents showed more dominant alleles for this trait. Therefore, the distribution of dominant and recessive alleles is not symmetrical which was confirmed in genetic component analysis in Table 2. These findings are in good agreement with those of Saleem et al. (2002), Watto et al. (2002), Betran et al. (2003), Prakash and Ganguli (2004), Ali et al. (2007), Irshad-ul-Haq (2010), Khodarahmpour (2011) and Hussain et al. (2014) for grain number and grain yield per plant under drought stress.

Potence ratio

The potence ratio (PR) of 28 F₁ crosses are shown in Table 3. The positive values ratio specified the degree of

Table 3. Potence ratio of 28 F₁ progeny of field corn.

Hybrids	DT	DS	ASI	EH (cm)	PH (cm)	NG/plant	100-GW(g)	Yield/plant (g)
CZ-28 × CZ-29	2.33	4.16	37.00	1.74	8.46	9.80	1.00	-15.77
CZ-28 × BIL-65	6.00	12.60	17.00	-1.26	0.40	4.13	-2.43	5.28
CZ-28 × CZ-36	1.37	2.00	5.40	-0.36	2.56	8.31	63.00	4.74
CZ-28 × CZ-12	4.27	6.00	13.00	2.31	7.04	54.90	13.63	79.45
CZ-28 × CZ-26	2.33	7.00	10.50	2.10	2.03	10.72	-2.00	53.80
CZ-28 × CZ-24	12.43	17.50	38.14	33.36	3.06	16.71	0.71	2.84
CZ-28 × 9MG	1.47	5.00	2.50	0.52	1.82	6.85	9.40	-29.73
CZ-29 × BIL-65	1.25	3.86	17.00	17.87	8.17	13.28	7.00	-35.13
CZ-29 × CZ-36	6.78	8.85	13.50	25.72	7.01	7.78	2.25	-50.53
CZ-29 × CZ-12	-7.57	9.89	18.00	14.01	3.80	21.99	9.09	7.31
CZ-29 × CZ-26	19.67	11.50	6.60	5.16	3.22	179.80	3.89	35.54
CZ-29 × CZ-24	4.38	6.07	92.33	0.96	2.03	-80.07	4.14	2.39
CZ-29 × 9MG	-75.00	8.00	0.56	1.33	1.14	29.84	3.67	30.36
BIL-65 × CZ-36	3.80	3.89	5.00	63.68	9.44	47.51	24.00	32.00
BIL-65 × CZ-12	7.67	14.20	0.50	22.78	3.36	5.32	29.91	-8.44
BIL-65 × CZ-26	6.54	11.67	-2.14	4.30	2.47	8.18	39.00	-7.40
BIL-65 × CZ-24	35.67	89.00	15.52	0.29	1.67	7.52	6.50	3.79
BIL-65 × 9MG	4.87	-18.00	-0.64	1.62	0.88	2.70	23.00	9.75
CZ-36 × CZ-12	3.00	4.82	9.67	15.28	6.23	4.78	41.00	3.34
CZ-36 × CZ-26	6.33	5.10	3.44	6.86	3.60	0.41	7.00	7.66
CZ-36 × CZ-24	3.09	3.29	4.58	2.14	2.70	-0.73	0.33	0.02
CZ-36 × 9MG	5.60	4.30	3.31	1.40	1.50	8.56	11.00	-3.11
CZ-12 × CZ-26	8.50	73.00	13.00	1.92	3.71	11.31	139.00	-22.07
CZ-12 × CZ-24	1.93	9.67	25.71	0.89	6.67	18.03	4.88	3.99
CZ-12 × 9MG	11.00	81.00	2.14	1.12	0.37	0.07	28.68	-15.77
CZ-26 × CZ-24	2.83	-8.43	5.68	2.92	37.07	86.60	12.60	2.31
CZ-26 × 9MG	40.00	-47.00	3.50	0.96	4.07	94.55	20.33	-318.00
CZ-24 × 9MG	4.78	-19.00	-3.53	0.42	4.22	28.62	-31.00	-8.60

DT=days to tasseling, DS=days to silking, ASI=anthesis-silking interval (days), PH=plant height (cm), EH=ear height (cm), NG/Plant=number of grains/plant and 100-GW=100-grain weight (g).

dominance ranging from partial to over-dominance. Again, negative values indicate the degrees of recessiveness ranging from partial to under recessiveness (Solieman et al., 2013).

For the trait DT, PR value ranged from -25 (CZ-29 × 9 MG) to 40 (CZ-26 × 9 MG). Among them no crosses showed complete dominance (-1.0) and all the 28 crosses exhibited over-dominance ($> \pm 1$). For the trait DS, the range of PR was -47.0 (CZ-26 × 9 MG) to 89 (BIL-65 × CZ-24) where all the crosses showed over-dominance ($> \pm 1$). The PR of the trait ASI was distributed from -3.53 to 92.33. Apart from two crosses, all other crosses were over dominant. In case of the trait EH, potence ratio spectrum is -1.26 (CZ-28 × BIL-65) to 63.68 (BIL-65 × CZ-36). Out of 28 crosses, seven (CZ-28 × CZ-36, CZ-28 × 9 MG, CZ-29 × CZ-24, BIL-65 × CZ-24, CZ-12 × CZ-24, CZ-26 × 9 MG and CZ-24 × 9 MG) showed partial dominance whereas the rest of the crosses showed over dominance (PR $> \pm 1$). PR for PH ranged from 0.37 (CZ-12

× 9 MG) to -37.07 (CZ-26 × CZ-24). Three crosses showed partial dominance while PR of the remaining crosses indicated over dominance. PR of the trait number of grain/plant ranges from -8.07 (CZ12 × CZ-24) to 94.55 (CZ-26 × 9 MG). The crosses CZ-36 × CZ-24, CZ-36 × CZ-26 and CZ-12 × 9 MG showed partial dominance. Other 25 crosses showed over dominance (PR $> \pm 1$). Considering the PR of 100 grain weight (100-GW), the range was -31.00 (CZ-24 × 9 MG) to 40.00 (CZ-36 × CZ-12). While one cross (CZ-28 × CZ-29) showed complete dominance (PR=1.00), two other crosses (CZ-36 × CZ-26) and (CZ-28 × CZ-24) showed partial dominance (PR<1.00); and all the other crosses showed over dominance (PR $> \pm 1$). In case of the last studied trait, yield/plant PR ranged from -47.00 (CZ-26 × 9 MG) to 79.45 (CZ-28 × CZ-12). The cross CZ-36 × CZ-24 showed absence of dominance for this trait. The rest of the crosses exhibited over dominant (PR $> \pm 1$). Previously, Begum et al. (2018) reported similar results

for different traits of maize under normal growing condition.

Conclusion

Genetic parameters, Vr-Wr and potence ratio suggested that the traits are governed by over dominance gene action. Study of the hybrids suggests that all the 28 cross combinations showed over dominance for the traits. However, the lower values of h^2_n indicated that the traits were highly influenced by the growing environment. Considering all of this, the data will be important in exploring heterosis breeding to improve those traits in maize under salinity. However, this type of result under salinity stress is not available. Therefore, further research in this circumstance can provide important information for developing saline tolerant varieties of crops.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Anther-derived callus induction based on culture medium, myo-inositol, AgNO₃ and Fe-EDTA in ‘Seolhyang’ strawberries

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Haploid breeding is an effective method of shortening the breeding period of plants. In this study, to develop a haploid breeding technique, optimum conditions of the culture medium were investigated for anther-derived callus induction in the strawberry cultivar Seolhyang. The effects of the culture medium type, myo-inositol, auxin and cytokinin combination treatment, silver nitrate (AgNO₃), and ferric ethylenediaminetetraacetic acid (Fe-EDTA) on anther-derived callus induction were analyzed. Anthers were incubated in Murashige and Skoog (MS) medium, Gamborg B5 medium (B5), and Lichter medium (NLN) for 8 weeks. Each culture medium had 0.4 mg·L⁻¹ of 6-benzyladenine (BA), 0.1 mg·L⁻¹ of indole-3 acetic acid (IAA), and 2.0 mg·L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) added to it. Results showed that MS medium was most effective in callus induction. When 100 mg·L⁻¹ of myo-inositol was added to each medium, the callus induction rate increased. Auxin and cytokinin combination treatment was more effective with the addition of 0.4 mg·L⁻¹ of BA, 0.1 mg·L⁻¹ of IAA, and 2.0 mg·L⁻¹ of 2,4-D to MS medium compared to the addition of 0.1 mg·L⁻¹ of BA, 2.0 mg·L⁻¹ of IAA, and 0.4 mg·L⁻¹ of 2,4-D. With AgNO₃ treatment, the highest callus induction rate was found at a concentration of 25 mg·L⁻¹. The callus induction rate increased as the AgNO₃ concentration increased; however, a significant decrease was seen at 30 mg·L⁻¹. In the case of Fe-EDTA, the most effective concentration for callus induction was 25 mg·L⁻¹. Therefore, supplementing myo-inositol, AgNO₃ and Fe-EDTA can help in anther-derived callus induction in Seolhyang strawberries.

Key words: Anther culture, auxin, B5 medium, callus induction, cytokinin, haploid breeding, NLN medium.

INTRODUCTION

Strawberries (*Fragaria x ananassa* Duch), a herbaceous perennial plant belonging to the Rosaceae family, are either consumed raw or used as a source material for jams, wines, juices, and other processed foods because of their sweetness and sourness together. Strawberries

are rich in antioxidants, vitamin C, anthocyanin and, and, therefore, function as a health food (Lilia et al., 2017). In South Korea, strawberry breeding and cultivation began in the 1970s. Since then, cultivars such as Josaenghongsim have been grown.

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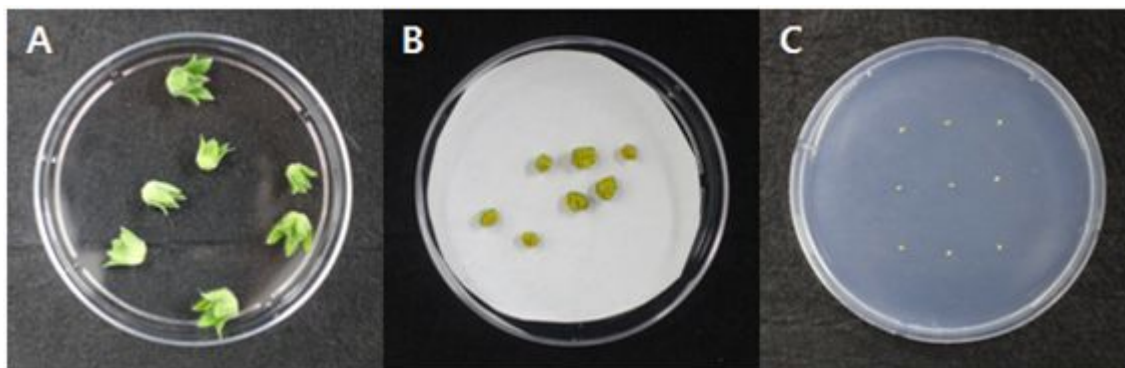


Figure 1. Experimental materials and process. (A) Closed bud, (B) closed bud with all petals removed, and (C) anther cultured in a Petri dish containing MS medium. MS, Murashige and Skoog.

However, the distribution of Josaenghongsim strawberries in farmhouses has considerably decreased compared to Japanese strawberry cultivars such as Red Pearl and *akihime*. In 2005, because of a royalty fee issue, the cultivar Seolhyang was developed in a strawberry research center. By 2015, the cultivation area for Seolhyang strawberries reached up to 80% of the entire strawberry cultivation area across South Korea. As the demand for strawberries increased in Southeast Asia, the strawberry exports continuously increased from \$4.2 million in 2004 to \$34 million in 2015. Seolhyang strawberries have high sugar content. In addition, they are resistant to powdery mildew, thus being convenient for cultivation as well as have a higher production efficiency compared to other cultivars. However, their texture is too soft, and they are easily spoiled and damaged when harvested. Therefore, they have low storability and cannot be exported (Reddy et al., 2000).

To meet the rising demand for strawberries in Southeast Asia, a high-quality strawberry cultivar with hard texture and good storability needs to be urgently developed. However, strawberries have high hyperdiploidy, that is, they are octoploids with 56 chromosomes. As a result, compared to other plants, inbred line production for strawberries is extremely difficult (Hirakawa et al., 2014). Therefore, it is desirable to develop haploid breeding techniques to save time and cost for the development of a new cultivar that meets the demands of the international market.

Few studies have been conducted on strawberry anther culture, although there are a few reports on meristem culture for virus-free strawberry production (Na et al., 2011; Nguyen et al., 2015). However, callus induction using anthers is an effective technique for strawberry haploid breeding. There are two ways of mass propagation using calluses, indirect embryogenesis and indirect organogenesis, both having a high proliferation efficiency (Niazian et al., 2017). In this study, optimal callus induction conditions were investigated using anthers in order to establish a haploid breeding technique

by examining suitable culture types and plant growth regulators for anther-derived callus induction and regenerated plant production in strawberries.

MATERIALS AND METHODS

Plant material

Seolhyang strawberry buds were collected from a strawberry farm in Okcheon-Myeon, Haenam-Gun, South Korea, in May 2016. The bud length (calyx to flower edge) was measured and buds with a length of 10 to 14 mm were selected; however, those with visible petal differentiation were excluded (Figure 1). The collected buds were incubated at 4°C for 4 days, disinfected in 70% ethanol for 15 s, and subsequently sterilized in 1% sodium hypochlorite for 15 min. Next, they were cleansed in sterilized water on a clean bench for 3 min; this was repeated thrice.

Callus induction

To examine the optimal chemical factor for anther culture, the following rates were measured: callus induction rate based on culture medium types of Murashige and Skoog (MS) medium, Gamborg B5 medium (B5), and Lichter medium (NLN); callus induction rate based on the combination of auxin and cytokinin in MS medium; and callus induction rate based on the addition of AgNO₃, ferric ethylenediaminetetraacetic acid (Fe-EDTA), and myo-inositol (Gamborg et al., 1968; Lichter, 1982; Murashige and Skoog, 1962). To investigate the optimal combination of auxin and cytokinin, callus formation was induced in MS1 medium (with 0.4 mg·L⁻¹ of 6-benzyladenine [BA], 0.1 mg·L⁻¹ of indole-3 acetic acid [IAA], and 2.0 mg·L⁻¹ of 2,4-dichlorophenoxyacetic acid [2,4-D] added) and MS2 medium (with 0.1 mg·L⁻¹ of BA, 2.0 mg·L⁻¹ of IAA, and 0.4 mg·L⁻¹ of 2,4-D added).

The callus induction rate in MS medium, B5, and NLN was investigated under the same conditions of plant growth-regulating substances. Equal amounts of plant growth-regulating substances (0.4 mg·L⁻¹ of BA, 0.1 mg·L⁻¹ of IAA, and 2.0 mg·L⁻¹ of 2,4-D) were added because these agents showed the best callus induction rate in preliminary tests. To determine the callus induction rate, anthers extracted from the buds were cultured in the dark in MS medium, B5, and NLN with equal amounts of BA, IAA, and 2,4-D, both with and without 100 mg·L⁻¹ of myo-inositol for 8 weeks.

To examine the effects of AgNO₃ and Fe-EDTA concentrations

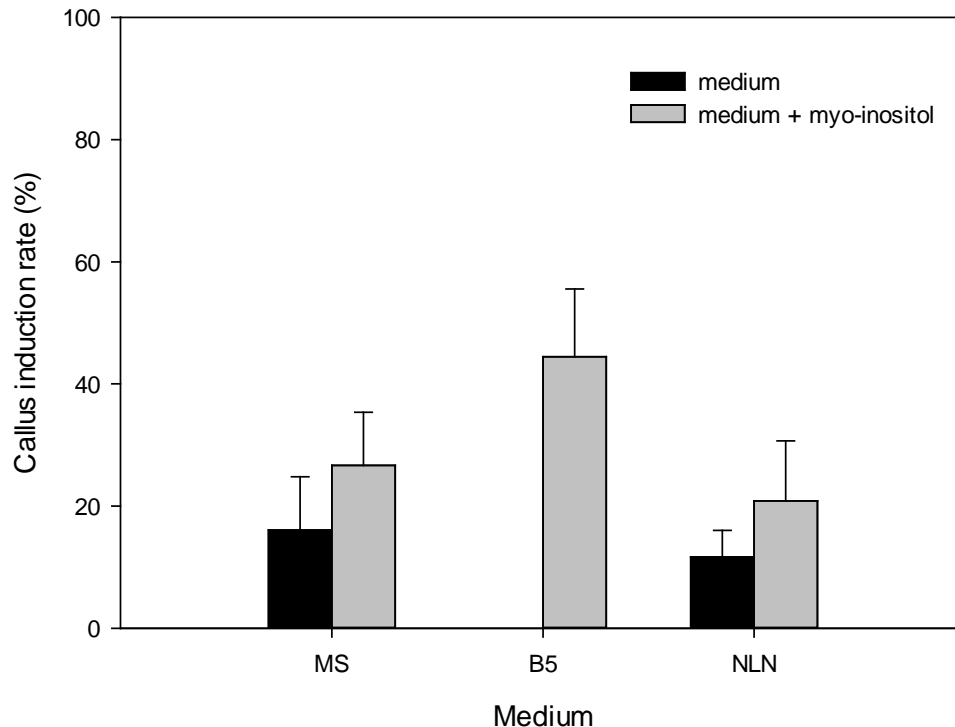


Figure 2. Effect of myo-inositol and different types of culture medium on anther-derived callus induction in Seolhyang strawberry anther culture.

on callus induction, 0, 5, 15, 25, and 30 mg·L⁻¹ of AgNO₃ and 0, 25, 50, 75, and 100 mg·L⁻¹ of Fe-EDTA were added to MS medium separately and cultured in the dark for 8 weeks before the callus induction rate was examined. In addition to 0.4 mg·L⁻¹ of BA, 0.1 mg·L⁻¹ of IAA, and 2.0 mg·L⁻¹ of 2,4-D, 30 g·L⁻¹ of sucrose and 8 g·L⁻¹ of agar were also added to all culture media. After adjusting the pH of the culture media to 5.8, the media was autoclaved in a high-pressure sterilizer at 120°C and 1.5 atmospheric pressure. For all experiments, a 90 × 20 mm² Petri dish in which six anthers were treated was used. After heat-shock treatment at 32°C for 48 h in the dark, the culture was maintained in a dark room at 25°C for 8 weeks. Thereafter, the callus induction rate was calculated as a percentage of the number of calluses induced from the six anthers cultured in the Petri dish. All experiments were repeated 10 times. The SigmaPlot 12.0 program was used for statistical analysis.

RESULTS AND DISCUSSION

Callus induction based on culture medium type, myo-inositol, and plant growth regulators

On the basis of the culture medium type, cultures without myo-inositol showed the highest callus induction rate of 16% in MS medium as compared to 0% in B5 and 11.7% in NLN (Figure 2). On the other hand, the callus induction rate in cultures with 100 mg·L⁻¹ of myo-inositol was highest in B5 (44.4%), followed by MS medium (13.7%) and NLN (9.1%) (Figures 2 and 3). The results of this study were consistent with those conducted on other

plants. As a biomembrane component, myo-inositol metabolizes into UDP-xylose or UDP-glucuronic acid to be used for cell wall polysaccharide biosynthesis and combines with IAA to play an important role in cell growth regulation (Loewus and Murthy, 2000). Myo-inositol improves callus induction in immature embryos of turf-type tall fescue (Bai and Qu, 2001). Myo-inositol is also an essential factor for cell wall formation and the phosphate pathway in plants (Bohnert et al., 1996; Hegeman et al., 2001). Moreover, myo-inositol protects plants against salt stress and is suggested to function in salt tolerance in two major ways: to protect cellular structures from reactive oxidizers and to control the water pressure inside cells (Loewus and Murthy, 2000). In addition, myo-inositol acts as a growth enhancer *in vitro* and a carbohydrate source, which are good osmotica for sustained cell division (Azad et al., 2006; Eun et al., 2011). Therefore, myo-inositol is considered an important factor for increasing the callus induction rate in strawberry anther culture. Using 0.4 mg·L⁻¹ of BA, 0.1 mg·L⁻¹ of IAA, and 2.0 mg·L⁻¹ of 2,4-D resulted in increasing the callus induction rate up to 33.3%, which is higher than the callus induction rate of 12.7% obtained with 0.1 mg·L⁻¹ of BA, 2.0 mg·L⁻¹ of IAA, and 0.4 mg·L⁻¹ of 2,4-D (Figure 4).

The auxin and cytokinin combined treatment is a way to increase the callus induction rate. Studies have reported that the callus induction rate is higher if the concentrations

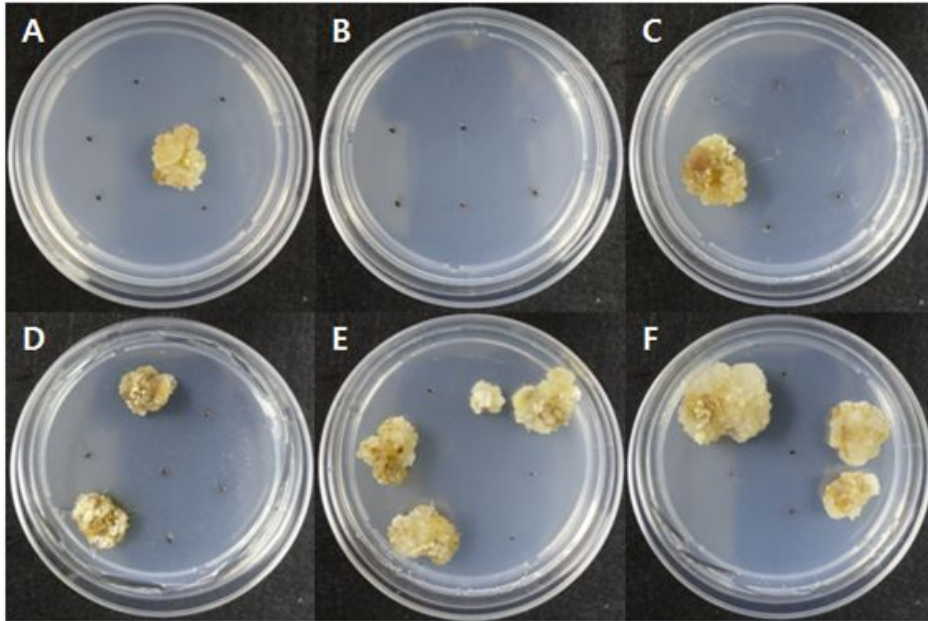


Figure 3. Morphology of anther-derived callus induction on solid media. Callus induced in a Petri dish containing (A) MS medium, (B) B5, (C) NLN, (D) MS medium + $100 \text{ mg}\cdot\text{L}^{-1}$ of myo-inositol, (E) B5 + $100 \text{ mg}\cdot\text{L}^{-1}$ myo-inositol, and (F) NLN + $100 \text{ mg}\cdot\text{L}^{-1}$ myo-inositol. MS, Murashige and Skoog; B5, Gamborg B5 medium; NLN, Lichter medium.

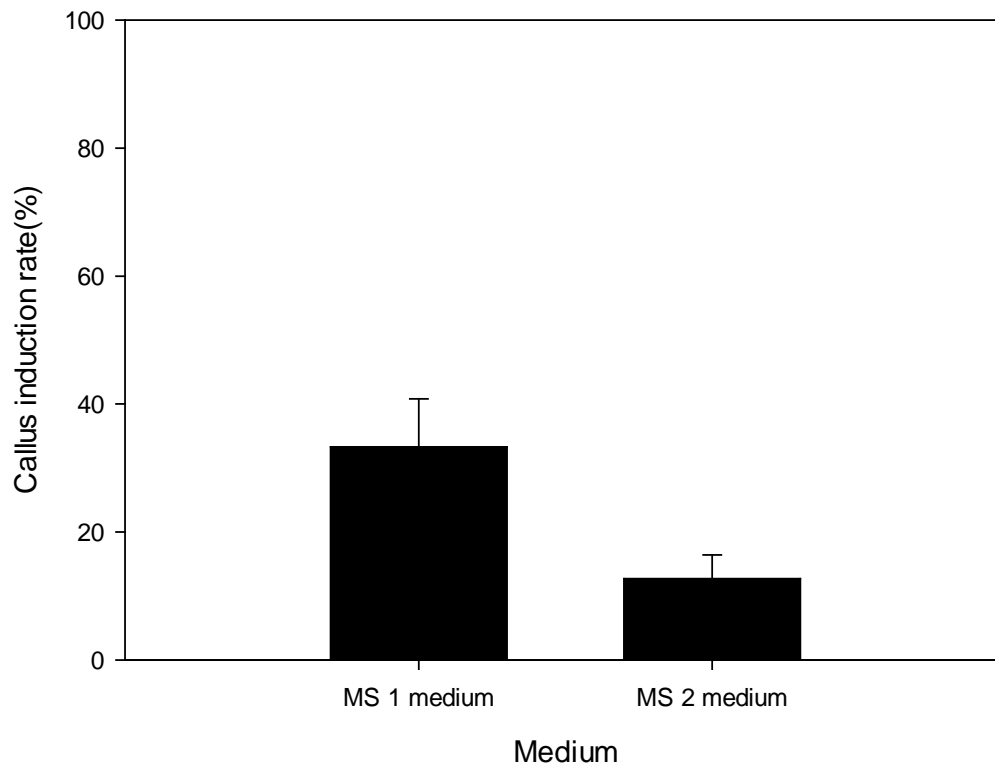


Figure 4. Effect of plant growth regulators on anther-derived callus induction in Seolhyang strawberry anther culture. MS1 medium was supplemented with $0.4 \text{ mg}\cdot\text{L}^{-1}$ of BA, $0.1 \text{ mg}\cdot\text{L}^{-1}$ of IAA, and $2.0 \text{ mg}\cdot\text{L}^{-1}$ of 2,4-D, and MS2 medium was supplemented with $0.1 \text{ mg}\cdot\text{L}^{-1}$ of BA, $2.0 \text{ mg}\cdot\text{L}^{-1}$ of IAA, and $0.4 \text{ mg}\cdot\text{L}^{-1}$ of 2,4-D. MS, Murashige and Skoog; BA, 6-benzyladenine; IAA, indole-3 acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

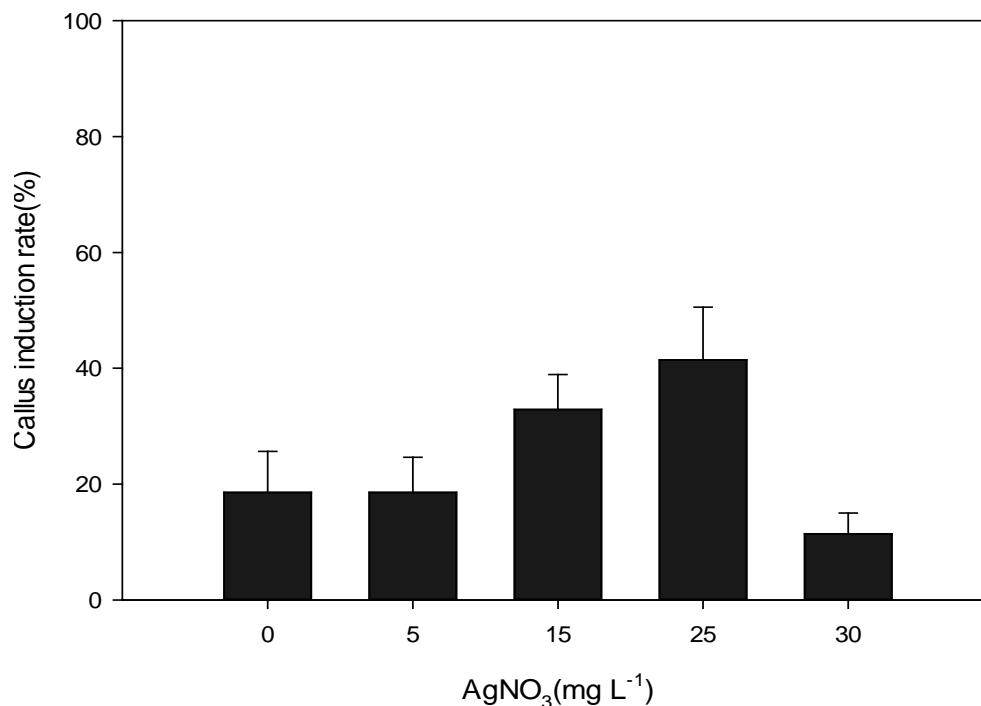


Figure 5. Effect of AgNO₃ concentration on anther-derived callus induction in Seolhyang strawberry anther culture.

of auxin and cytokinin are the same in *Muscari comosum* var. *Plumosum* (He et al., 2006). However, a culture medium with a higher concentration of 2,4-D than BA is more suitable for *Callerya speciosa* anther culture (family Fabaceae) (Huang et al., 2016). The callus induction rate with combined auxin and cytokinin treatment varies depending on plant varieties as well as the type and concentration of plant growth regulators.

Callus induction based on AgNO₃ and Fe-EDTA

On the basis of the AgNO₃ concentration, the callus induction rate was highest at 41.4% with 25 mg·L⁻¹ of AgNO₃, whereas the callus induction rates in cultures with 5 and 15 mg·L⁻¹ of AgNO₃ were 18.6 and 32.9%, respectively, confirming that the callus induction rate increases with the AgNO₃ concentration (Figure 5). However, the callus induction rate rapidly decreased to 11.43% with 30 mg·L⁻¹ of AgNO₃. AgNO₃ inhibits cell aging by inhibiting ethylene generation in ethylene receptors. Therefore, AgNO₃ is reportedly effective for long-term callus cultures because it inhibits ethylene generated by aging and stress (Williams et al., 1990). Explants in culture media supplemented with a low concentration of AgNO₃ improved the embryogenic callus size and texture, whereas higher concentrations of AgNO₃ decreased the embryogenic callus induction rate. In addition, in *Solanum nigrum* (L.), AgNO₃ significantly

induced more embryogenic calluses compared to AgNO₃-free culture media (Geetha et al., 2016). For strawberry callus culture, the results of this study confirmed that AgNO₃ is effective for callus maintenance and culture.

In this study, it was also observed that 25 mg·L⁻¹ of Fe-EDTA resulted in the highest callus induction rate at 28.6%. With 0, 50, 75 and 100 mg·L⁻¹ of Fe-EDTA, the callus induction rate was 16.1, 21.4, 15.8 and 18.6%, respectively. A Fe-EDTA concentration of >50 mg·L⁻¹ showed no effect, because the callus induction rate was similar to cultures without Fe-EDTA treatment (Figure 6). In orchid cultures, Fe-EDTA is effective in callus induction and protocorm-like body formation (Silva et al., 2006). As a source of iron, Fe-EDTA is used in tissue culture of many plants because it prevents a decrease in IAA activity by photochemical reactions (Hangarter and Stasinopoulos, 1991). In this study, we found that adding an appropriate concentration of Fe-EDTA to MS medium can effectively increase callus induction.

Conclusion

Supplementing BA (0.4 mg·L⁻¹), IAA (0.1 mg·L⁻¹), 2,4-D (2.0 mg·L⁻¹), myo-inositol (100.0 mg·L⁻¹), AgNO₃ (25.0 mg·L⁻¹), and Fe-EDTA (50.0 mg·L⁻¹) in B5 can help in anther-derived callus induction in Seolhyang strawberries. This study identified the optimal conditions for haploid breeding of Seolhyang strawberries, which can also be

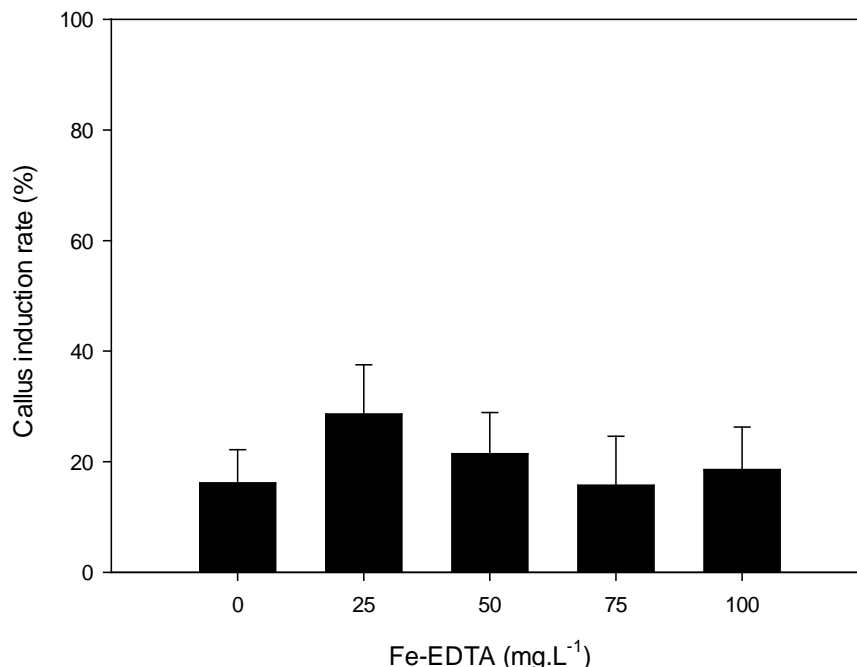


Figure 6. Effect of Fe-EDTA concentration on anther-derived callus induction in Seolhyang strawberry anther culture. Fe-EDTA, ferric ethylenediaminetetraacetic acid.

applicable for the cultivation of other strawberry cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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