Theor Appl Genet (2003) 106:771–776
DOI 10.1007/s00122-002-1143-6

C. Sreekala · S. P. S. Raghava

Exploitation of heterosis for carotenoid content in African marigold (*Tagetes erecta* L.) and its correlation with esterase polymorphism

Received: 4 April 2002 / Accepted: 23 July 2002 / Published online: 30 October 2002

Abstract African marigold (*Tagetes erecta* L.), a major source of carotenoids, is also grown as a cut flower and a garden flower in addition to being grown for its medicinal values. We studied gene action, combining ability and heterosis, aiming at genetic improvement of *T. erecta* for enhanced carotenoid content in petals, and report for the first time that heterosis can be exploited for total carotenoids and its commercially important fractions. Total content of carotenoids and lutein appears to be governed by dominance (or non-additive) gene action, while content of xanthophyll esters is governed by both additive and dominance (or non-additive) gene actions. Specific combining ability variance was predominant for all the three traits. General and specific combining abilities and heterosis were highly significant. Heterobeltiosis was also positive. General combining ability (GCA) variances were not significantly correlated to performance per se. There was also no correlation between performance per se of normal petalled pollen parents and the performance of crosses made between male-sterile (female) and male-fertile (pollen) parents. These findings suggest that carotenoid content should not be the only criterion considered in the selection of parental lines. Studies on esterase in seeds and peroxidase in seedlings revealed a relatively high level of polymorphism in esterase with a total of 14 isoforms, whereas peroxidase showed low polymorphism. Similarity indices between different parental combinations, calculated based on seed esterase polymorphism, showed a significant negative correlation ($r = –0.479$, $P = 0.05$) with heterosis for carotenoid content. This indicates that the selection of parents with wider variation in their esterase profiles may possibly be exploited for genetic enhancement of carotenoids in *T. erecta*.

Keywords Carotenoids · Combining ability · Gene action · Heterosis · Isozymes · Lutein · Xanthophyll esters

Communicated by H. Nybom

C. Sreekala (✉) · S.P.S. Raghava
Division of Floriculture and Landscaping,
Indian Agricultural Research Institute,
New Delhi-110 012, India
e-mail: sreekala@tll.org.sg, Fax: +65-6872-7007

Present address:
C. Sreekala, Laboratory of Molecular Plant Pathology,
Temasek Life Sciences Laboratory, IMA Building, 1,
Research Link, National University of Singapore,
Singapore-117 604

Introduction

African marigold (*Tagetes erecta* L.) is an economically important ornamental that is grown as a cut-flower crop, as a garden flower and for its medicinal values. It belongs to the family Asteraceae and is native to the South and Central Americas, especially Mexico. Pigments from this plant have various physiological and pharmacological uses. Carotenoids extracted from *T. erecta* find applications in poultry feed as additives to enhance chicken skin and egg yolk coloration (Scott et al. 1968) at a considerably lower cost than synthetic or other natural carotenoids (Seemann 1998). Purified xanthophyll esters are useful as an ophthalmologic agent (Gau et al. 1983) and also as a potential food-coloring agent since it is a concentrated source of carotenoids (Timberlake and Henry 1986). Lutein is the major xanthophyll (70–88%) present in the petals of *Tagetes* spp. (Quackenbush and Miller 1972; Hadden et al. 1999; Sreekala 2000), which normally occur as palmitic and myristic acylates (Karawya et al. 1996). Also, lutein fatty acid esters from *T. erecta* are readily soluble in vegetable oils as compared to other FDA approved synthetic carotenoids (Timberlake and Henry 1986), which makes them readily useable in several industrial applications. Dietary carotenoids are also used in the treatment of cancer and other photosensitivity diseases (Gau et al. 1983; Park et al. 1998). Furthermore, this crop can be cultivated under almost all known climatic conditions. Hybrid varieties of *T. erecta* are being cultivated globally for ornamental purposes and pig-

ment extraction, but carotenoid content was, however, not considered in their development. Genetic improvement of this crop should, therefore, include breeding strategies designed at improving the carotenoid content. The existence of previously known male-sterile lines in this crop makes hybridization attempts comparatively simple and cheap.

In contrast, breeding for carotenoid content has been studied in great detail in other crops such as tomato (Rao and Choudhary 1974; Bhutani and Kalloo 1983; Amaral et al. 1997), tulips (Nieuwhof et al. 1988) and camellia (Hwang et al. 1992). On the basis of a study using a diallel crossing design involving seven cultivars of tulips, Nieuwhof et al. (1988) reported that carotenoid content was governed by non-additive gene action, whereas another study on spring wheat (*Triticum durum*) showed that the grain carotenoids were controlled by genes with additive effects (Bebyakin and Starichkova 1992). However, a study on tomato showed that both additive and dominance (or non-additive) gene action occurred in the same crop (Ognyanova and Moinova 1973).

Plant total carotenoids consist of several fractions, which include different carotenes (α, β and γ fractions), xanthophylls (lutein, zeaxanthin) and their esterified forms (Goodwin 1965). Observations from the abovementioned studies indicate that each of the component fractions may be controlled by a different type of gene action. Crosses involving genotypes with varying carotenoid compositions may therefore yield different results due to variations in the composition of the carotenoid fractions. Consequently, gene action, combining ability and heterosis for each of the commercially important fractions need to be studied in detail. Isozymes come in as handy tools to identify diversity amongst genotypes (Swiecicki and Wolko 1987; Moosgos and Friedt 1992). In certain cases, information on the diversity obtained using isozymes can be correlated directly with expression of heterosis (Chunlin 1996). We report here the findings of a study on gene action, combining ability, heterosis and the most suitable methodology for genetic improvement of carotenoids in *T. erecta* using conventional breeding practices.

Materials and methods

Materials

Three male-sterile lines of *Tagetes erecta* – numbered MS_5 (yellow), MS_7 (light orange) and MS_8 (deep orange) – having visual variations in their stigma color were used as female parents. These lines were derived from the F_1 hybrids produced by using the apetalous type (femina) of male sterility. Six inbred lines, Sel-19, Sel-22, Sel-29, Sel-51, Sel-63 and FM-560, were used as pollen parents. These lines were developed as a result of individual plant selections from the heterozygous populations followed by inbreeding until the homozygosity stage. An additional, open-pollinated variety was also used as a pollen parent, namely, Pusa Narangi Gainda (PNG), which was developed through eight generations of selection from the initial cross between the varieties Cracker Jack and Golden Jubilee.

Testing for heterosis, combining ability and gene action

Apetalous male-sterile plants were used as female parents and normal flowered plants as pollen parents. A line (female) × tester (male) mating design (Kempthorne 1957) was adopted. An assessment of variability for carotenoids amongst parental materials has previously been carried out (Sreekala et al. 2003), and the results revealed high variability among all ten parental lines.

Parents were grown at a normal spacing of 45 × 45 cm under optimal growth conditions. Before flowering, both male and female parents were bagged with muslin cloth bags to prevent contamination with unwanted pollen. Hybridization was carried out by dusting pollen from the male parents onto the styles of the male-sterile flower as the latter emerged out of the bud. The most suitable time for successful hybridization was observed to be between 10 a.m. and 1 p.m. Hybridization was repeated three to four times on a single flower till the flowers were fully open. The 21 hybrids obtained from crosses involving three male-sterile lines with seven pollen parents were evaluated in a randomized block design (RBD) with three replications. Flowers were harvested at the full bloom stage and analysed for carotenoids as described below. Statistical analysis for a line × tester mating design was carried out for carotenoids using the computer programme SPAR1 (Statistical Programme for Agricultural Research, Indian Agricultural Statistics Research Institute, New Delhi, India) that was designed according to the procedure of Kempthorne (1957).

Estimation of total carotenoids, xanthophyll esters and lutein

Total carotenoids, xanthophyll esters and lutein contents were estimated as previously described (Quackenbush et al. 1970; Quackenbush and Miller 1972; Philip and Berry 1976; Ranganna 1995) with slight modifications. Fresh flowers were selected for analysis, and the total carotenoids were estimated using the method suggested by Ranganna (1995). Subsequently, the total carotenoids were subjected to saponification that aids in conversion of xanthophyll esters to free xanthophylls, as suggested by Quackenbush et al. (1970). Column chromatography was performed for both saponified and non-saponified samples (for both dry and fresh flowers) as described (Quackenbush and Miller 1972) to estimate total and free xanthophylls. The difference between total and free xanthophylls yield the xanthophyll esters present in these flower samples. However, a slight modification was adopted by using an alumina (aluminium oxide) column instead of a MgO-supercel column as described in the original procedure. Results obtained for both dry and fresh flowers were comparable with previous findings (Verghese 1996).

Isozyme analysis

Isozyme analyses of esterase (using seed samples) and peroxidase (using 6-week old seedlings) were performed. Isozyme patterns of the parental genotypes were analysed for esterase and peroxidase enzyme systems using standard procedures (Sadasivam and Manickam 1996) with slight modifications in the extraction of enzymes. The extraction buffer used for seed samples (for esterase) was a borate buffer (50 m*M*), pH 8.7. A Tris-HCl buffer pH 8.7, containing 114 m*M* NaCl, 5 m*M* ascorbic acid and 20 g/l PVP was used for extracting peroxidase enzyme from the seedlings. A pinch of PVPP was also added while grinding the sample. Similarity indices (Nei and Li 1979) were calculated from the obtained band profiles for each parental combination (e.g., between MS_5 and Sel-19, MS_5 and PNG, etc., to MS_8 and FM-560). Values of similarity indices for all 21 parental combinations were analysed for possible correlation with level of heterosis for carotenoid content in all the corresponding 21 hybrids.

Results and discussion

Combining ability and heterosis

Analysis of variance for combining ability (Table 1) shows that female versus male mean squares were highly significant for total carotenoids, xanthophyll esters and lutein. A significant interaction was observed between the lines and testers, indicating that the male-sterile lines did not behave consistently with the testers and vice versa. Mean squares for male-sterile lines were found to be significant only in the case of xanthophyll esters.

General combining-ability (GCA) effects of parents showed that MS_8 was the best general combiner among the male-sterile lines. It showed a highly significant positive GCA effect for total carotenoids (Table 2), xanthophyll esters (0.38***, data not shown) and lutein (0.20**, data not shown) content, whereas Sel-29 was the best general combiner amongst all the pollen parents for total carotenoids (Table 2) and xanthophyll esters (0.27**, data not shown) and had a significant and positive GCA for lutein (0.09*, data not shown). Sel-51 showed the highest GCA (0.37**, data not shown) for lutein amongst all the pollen parents. Many hybrids generated in this study showed a highly significant specific combining-ability (SCA) effect (Table 2). The above results indicate a substantial potential to manipulate carotenoid content in *T. erecta* by conventional breeding methods. Additionally, highly significant variability between female and male parents for all the carotenoid fractions (Table 1) highlights the possibility of genetic improvement of the corresponding traits. Similar highly significant differences among F_1 hybrids for carotenoids were previously reported in tulips (Nieuwhof et al. 1988).

A number of hybrids showed highly significant heterosis, reaching up to 125.72% in $MS_8 \times$ Sel-29 for total carotenoid content (Table 3), but heterosis was demonstrated also for xanthophyll esters and lutein (data not shown). We observed high heterobeltiosis [increased performance of the hybrid over the better parent, (Fonseca and Patterson 1968)] for total carotenoids (Table 3), xanthophyll esters and lutein (data not shown) in a few hybrids. Significant heterosis and highly significant SCA effects were observed in a cross involving $MS_8 \times$ Sel-29 for total carotenoids, xanthophyll esters and lutein content. High mean performance for total carotenoids, high heterosis, positive heterobeltiosis and highly significant SCA values are all indicative of the commercial potential of this hybrid ($MS_8 \times$ Sel-29). In addition, both of its parents also showed high GCA in the desired direction

Table 1 Analysis of variance for combining ability for total carotenoids, xanthophyll esters and lutein content

Source	*df*	Mean squares		
		Total carotenoids	Xanthophyll esters	Lutein
Replication	2	1.9060**	0.0783	0.1343*
Females	2	2.1841	2.5457*	0.5991
Males	6	0.5700	0.3495	0.4118
Females vs. males	12	0.9564**	0.5191**	0.4733**
Error	40	0.2777	0.1577	0.0303

*, ** Significant at 5% and 1% level, respectively

Table 2 The GCA and SCA effects of parents[a] and hybrids[a] for total carotenoid contents (*SE* Standard error)

Testers (males)	SCA effects of hybrids							GCA effects of lines
	Sel-19	PNG	Sel-22	Sel-29	Sel-51	Sel-63	FM-560	
Lines (females)								
MS_5	–0.01	–0.71**	0.55*	–0.37	0.23	–0.43*	0.77**	0.11
MS_7	0.10	0.37	–0.70**	–0.09	0.34	0.08	–0.10	–0.25**
MS_8	–0.09	0.34	0.15	0.48**	–0.57**	0.35	–0.67**	0.36***
GCA effects of testers	0.09	0.01	–0.42**	0.39**	0.04	–0.19	0.09	

*, ** Significant at 5%, 1% and 0.1% level, respectively
[a] SE of lines: 0.08, SE_D: 0.23; SE of testers: 0.13, SE_D: 0.15; SE of hybrids: 0.19, SE_D: 0.40

Table 3 Expression of heterosis and heterobeltiosis in hybrids for total carotenoid content

Testers (males)	Sel-19		PNG		Sel-22		Sel-29		Sel-51		Sel-63		FM-560	
Lines (females)	di[a]	dii[b]	di	dii	di	dii	di	dii	di	dii	di	dii	di	dii
MS_5	79.00*	–5.60	20.99	–36.60	96.12*	3.78	80.39*	–4.76	68.69	–11.64	57.40	–16.50	90.39*	55.54
MS_7	68.58	–6.38	61.10	–11.49	4.53	–41.47	80.13*	0.41	59.36	–12.52	70.28	–4.18	101.84*	–22.75
MS_8	76.05	6.28	75.51	4.01	73.54	6.26	125.72**	37.16	35.02	–20.11	103.04**	25.00	84.68*	17.68

*, **, *** Significant at 5%, and 1% level, respectively
[a] di, Heterosis; SE = 0.378
[b] dii, Heterobeltiosis; SE = 0.436

Table 4 Genetic components of variance for total carotenoids, xanthophyll esters and lutein

Source of variance	Total carotenoids	Xanthophyll esters	Lutein
GCA	0.028	0.062	0.035
SCA	0.226	0.120	0.149
GCA/SCA	0.124	0.517	0.235

Table 5 Correlation between genetic parameters and performance per se[a] of pollen parents for total carotenoids, xanthophyll esters and lutein

Analysed combinations of variables	Total carotenoids	Xanthophyll esters	Lutein
SCA and heterosis	0.778**	0.605**	0.780**
GCA and parent per se	0.067	–0.081	0.165
Performance per se and hybrid performance of pollen parents	0.119	–0.336	0.368

** Significant at 1% level
[a] Performance, per se, refers to the mean performance of a genotype without the influence of the other genome in a particular cross

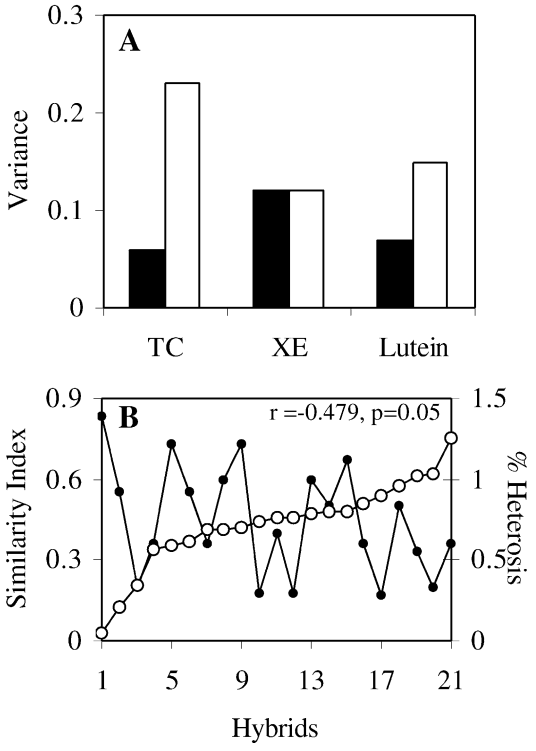

Fig. 1 **A** Additive and dominance variance for carotenoids at the full bloom stage. *TC* Total carotenoids, *XE* xanthophyll esters, (solid bar) additive variance. (open bar) dominance variance. **B** Similarity indices based on seed esterase band profiles for the parental combinations and percentage heterosis for total carotenoid content in their offspring. (Open circles) percentage heterosis, (solid circles) similarity index

(0.36*** for MS_8; 0.39** for Sel-29, as in Table 2), which makes the hybrid MS_8 × Sel-29 the material of choice for further breeding programmes.

Our study of combining ability variances (Table 4) shows that SCA variance was predominant for total carotenoids (0.226), xanthophyll esters (0.120) and lutein (0.149). The GCA/SCA ratio was also below unity for all of the three groups studied, which demonstrates the predominance of non-additive gene action for the above three traits. Therefore, the exploitation of heterosis for a higher carotenoid content appears promising in *T. erecta*. It can also be noted that the GCA/SCA ratio was largest for xanthophyll esters (0.517, as shown in Table 4). Further computation of additive and dominance variance showed that the latter was invariably predominant for total carotenoids and lutein (Fig. 1A), indicating non-additive gene action. Previous studies have shown that chilli peppers (Lippert 1975), tomato (Bhutani and Kalloo 1983) and tulips (Nieuwhof et al. 1988) also showed dominance (non-additive) gene action for carotenoids. As far as xanthophyll esters are concerned, additive and dominance variance were observed to be almost equal (Fig. 1A), indicating that selection for genetic improvement as well as exploitation of heterosis should be possible. Additionally, a high GCA/SCA ratio for xanthophyll esters (0.517, as in Table 4) also leads to the same conclusion as above. As dominance variance and, therefore, dominance (or non-additive) gene action exists for all the three fractions, exploitation of heterosis seems to be the most suitable method to achieve an enhanced production of carotenoids. Also, the production of F_1 hybrids for higher total carotenoids holds promise in the improvement of all the commercially important carotenoid fractions.

Values for SCA and heterosis (Table 5) showed highly significant correlation, but performance per se of parents was not correlated with GCA. In addition, the performance per se of pollen parents was also observed to lack significant correlation with the performance of crosses. These observations lead to the conclusion that performance per se of parents should not be considered as a criterion for the selection of parents in breeding exercises, which is in agreement with the basic theory that performance per se should not be regarded as an indicator of high combining abilities (Dabholkar 1992).

Polymorphism in isozymes

In the present study, 14 isoforms (observed as 14 bands representing different molecular forms of one enzyme) of seed esterase were identified, suggesting high levels of polymorphism for seed esterase in the parental lines of *T. erecta* (Fig. 2). We observed that marigold seeds failed to test positive for peroxidase enzyme activity, whereas three-WAS (3 weeks after sowing)-old seedlings showed slight peroxidase activity. However, with progress in time, an increase in activity was observed and

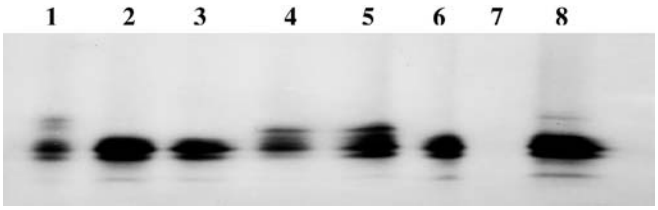

Fig. 2 Seed esterase pattern for selected parental lines. *Lane 1* MS_7, *lanes 2*, *3*, MS_8, *lanes 4*, *5* Sel-19, *lanes 6*, *8* PNG

six-WAS-old seedlings showed a remarkable increase in peroxidase activity. However, the level of polymorphism was low, with only four detectable bands (data not shown).

Similarity indices were calculated between different parental combinations involving both the esterase and peroxidase banding patterns. These indices were analysed for possible correlation with level of heterosis for carotenoid content in the respective hybrids. For seed esterase, a significant negative correlation (r = –0.479, p = 0.05, data not shown) was observed (Fig. 1B). This indicates that the greater the diversity is, as assessed through seed esterase band profiles, the better are the chances of exploitation of heterosis for carotenoid content. Similarly, in tea, hybrids with larger zymogram differences (or greater diversity) were reported to be highly heterotic (Chunlin 1996). The higher level of polymorphism (14 isoforms) seen in seed esterase is an indication of the existence of a sufficient number of alleles that makes esterase more reliable than peroxidase in the estimation of similarity indices between parents in *T. erecta*.

The results of the investigation reported here indicate that genetic improvement of carotenoid content in *T. erecta* should be feasible. We report here for the first time that exploitation of heterosis should be useful in breeding for increased carotenoid levels in addition to being helpful in improving the content of other commercially important fractions, such as xanthophyll esters and lutein. Promising parental combinations can now be identified using seed esterase profiles in *T. erecta*. It is possible that, interspecific hybridization between *T. erecta* and *T. patula* could also be exploited, since it is likely to yield triploids with a very high content of carotenoids. Additionally, knowledge of the carotenoid biosynthetic pathway and enzymes involved, identification of 17 different carotenoid fractions in *T. erecta*, and previously established gene transformation technology should be useful in initiating molecular flower breeding exercises resulting in large-scale synthesis of different carotenoids, including dietary carotenoids, from *Tagetes* spp. for commercialization.

References

Amaral Jr AT, Casali VWD, Cruz CD, Finger FL, Scapim CA (1997) Tomato breeding: II Gardner and Eberhart's methodology analysis of heterosis related to morphological characteristics and fruit quality. Bragantia 56:33–46

Bebyakin VM, Starichkova NI (1992) Genetic analysis of the content of carotenoid pigments in the grain of spring durum wheat. Tsitol Genet 26:52–57

Bhutani RD, Kalloo G (1983) Genetics of carotenoids and lycopene in tomato (*Lycopersicon esculentum* Mill.). Genet Agrar 37:1–6

Chunlin C (1996) Analysis of tea plants F_1 hybrids. J Tea Sci 16: 31–36

Dabholkar AR (1992) Elements of biometrical genetics. Concept, New Delhi

Fonseca S, Patterson FL (1968) Hybrid vigour in a seven parent diallel cross in common wheat (*Triticum aestivum* L). Crop Sci 8:85–88

Gau W, Ploschke HJ, Wünsche C (1983) Mass spectrometric identification of xanthophyll fatty acid esters from marigold flower (*Tagetes erecta*) obtained by high performances liquid chromatography and craig counter-current distribution. J Chromatogr 262:277–284

Goodwin TW (1965) Chemistry and biochemistry of plant pigments. Academic, London New York

Hadden WL, Watkins RH, Levy LW, Regalado E, Rivadeneira DM, van Breemen RB, Schwartz SJ (1999) Carotenoid composition of marigold (*Tagetes erecta*) flower extract used as nutritional supplement. J Agric Food Chem 47:4189–4194

Hwang YJ, Miyajima I, Okubo H, Fujieda K (1992) Flower colours and pigments of interspecific hybrids between *Camellia japonica* L. and *C. chrysantha* (Hu) Tuyama. J Fac Agric Kyushu Univ 36:165–172

Karawya MS, Hammouda FM, Ismail SI, Zaki AK, Nasif NM (1996) HPLC and MS analysis of lutein esters from marigold (*Tagetes patula* L). Qatar Univ Sci J 16:251–255

Kempthorne O (1957) An introduction to genetic statistics. John Wiley and Sons, New York

Lippert LF (1975) Heterosis and combining ability in chilli peppers by diallel analysis. Crop Sci 15:323–325

Moosgos G, Friedt W (1992) Comparison of isozyme electrophoresis and molecular methods in screening of near isogenic sunflower lines for resistance genes. Proc 13th Int Sunflower Conf, vol 2, pp 1485–1491

Nei M, Li W (1979) Mathematical model for studying genetic variations in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–573

Nieuwhof M, Keijzer P, Eikelboom W, van Eijk JP (1988) Inheritance of flower pigments in tulip (*Tulipa* L.). Euphytica 38:49–54

Ognyanova A, Moinova K (1973) Inheritance of beta-carotene content in red and orange-fruited tomatoes. Genet Sel 6:3–17

Park JS, Chew BP, Wong TS (1998) Dietary lutein from marigold extract inhibits mammary tumor development in BALB/c mice. J Nutr 128:1650–1656

Philip T, Berry JW (1976) Nature of lutein acylation in marigold (*Tagetes erecta*) flowers. J Food Sci 40:1089

Quackenbush FW, Miller SL (1972) Composition and analysis of the carotenoids in marigold petals. J Assoc Off Agric Chem 55:617–621

Quackenbush FW, Dyer MA, Smallidge RL (1970) Analysis of carotenes and xanthophylls in dried plant materials. J Assoc Agric Chem 53:181–185

Ranganna S (1995) Handbook of analysis and quality control for fruit and vegetable products 2nd Tata McGraw Hill, New Delhi

Rao RM, Choudhary B (1974) Inheritance of processing qualities of tomato (*Lycopersicon esculentum* Mill.). In: Proc 29th Int Hortic Congr I, Sect 8, Vegetables, pp 621–684

Sadasivam S, Manickam A (1996) Biochemical methods. Wiley Eastern and Tamil Nadu Agricultural University, Coimbatore, India

Scott MI, Ascarelli I, Olson G (1968) Studies on egg-yolk pigmentation. Poult Sci 47:863

Seemann M (1998) Latest trends in layer nutrition. How does the yellow get into the egg? Lohmann Inf 21:7–11

Sreekala C (2000) Production of F_1 hybrids in African marigold (*Tagetes erecta* L.) for higher content of carotenoids. PhD thesis, Post Graduate School, Indian Agricultural Research Institute, New Delhi, India

Sreekala C, Raghava SPS, Misra RL, Voleti SR (2003) Assessment of variability for carotenoids and yield components in African marigold. J Ornament Hortic 6, in press

Swiecicki WK, Wolko B (1987) Application of electrophoretic methods of isozyme separation to genetical characterization of pea (*Pisum sativum* L.) cultivars. Genet Pol 28:89–99

Timberlake CF, Henry BS (1986) Plant pigments as natural food colors. Endeavour 10:31–36

Verghese J (1996) Focus on xanthophylls from *Tagetes erecta* L. The giant natural colour complex-I. Indian Spices 33:8–13