Genetic-phytohormonal interactions in male fertility and male sterility phenotype expression in sunflower (*Helianthus annuus* L.)

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ABSTRACT

Amounts of indole-3-acetic acid (IAA) and gibberellic acid (GA_3) have been investigated in vegetative and reproductive tissues of diverse sunflower genotypes using gas-liquid chromatography. Quantification of endogenous GA_3 content from homozygote MB514 line, characterized by cytoplasmic male sterility (CMS), revealed a lower level in comparison to the fertility restorer RW637Rf line, which contained a higher hormone quantity. The largest amount of IAA was found in the heterozygote F_1 hybrid obtained by crossing these lines, regardless of the tissues and ontogenesis phases analyzed. Similar features were found in leaves, apex, inflorescence, and disc flowers in most of the variants investigated.

Key words: auxins – CMS-Rf system – gibberellins – *Helianthus annuus* L. – male fertility – male sterility.

INTRODUCTION

Genetic CMS-Rf system (*cytoplasmic male sterility – fertility restoration of pollen*) is a well known and versatile phenomenon that has been the subject of many studies due to its importance in commercial hybrid breeding and heterosis (Vrânceanu and Stoenescu, 1971; Voscoboinik, 1977). Besides, this genetic system represents a useful model for revealing nucleus-cytoplasm interaction mechanisms in male sterility-fertility expression. It has been shown that CMS in sunflower is associated with mitochondrial gene *orfH522* (Laver et al., 1991; Horn et al., 1994) that can be suppressed in F_1 hybrids based on CMS by the action of nuclear-encoded fertility restorer Rf genes both in homo – and heterozygous condition (Vrânceanu and Stoenescu, 1971; Anascenco and Duca, 1985). Also, male sterility can be induced by gibberelic acid (GA_3) treatment of plants and the same class of phytohormones restores male fertility to sterile plants (Anascenco, 1971). These phenomena sustain the hypothesis that plant hormones regulate the nucleus and other cell structure activity by the induction or suppression cytoplasmic systems of genes expression (Collett at al., 2000). It is assumed that the phenotype expression of hereditary male fertility/sterility trait is regulated by the phytohormones.

In the present research, the quantification of IAA and GA_3 endogen levels has been studied in several sunflower genotypes during their ontogenesis to reveal the interaction between genetic (nuclear and mitochondrial) factors and phytohormones in CMS–Rf phenotype expression and how these relationships influence the physiological and biochemical basis of microsporogenesis. Also, we studied two functional states of male gametophyte (male sterility/fertility) in the same nuclear context, using the phenocopies method, to obtain information on nuclear influence on the mitochondrial genome expression related to the CMS- Rf genetic system.

MATERIALS AND METHODS

Plant materials

Two sunflower isonuclear lines distinguished only by cytoplasm genes (MB514 and MB514CMS with mitochondrial *orfH522*), the line RW637Rf with nuclear homozygote restoration nuclear gene *Rf*, and the F_1 hybrid obtained by cross between these lines (MB 514 CMS x RW637Rf) with restored male fertility (*Rf_*) were used in this study. The plants were cultivated in the experimental field of Moldova State University according to conventional technologies during four years. Sunflower seeds were kindly provided by SRC ``Magroselect`` (Soroca, Republic of Moldova).

The apex decapitation at two leaves stage is a good way to obtain phenocopies, known as being convenient models for functional activity studies of a gene. As a result of apical domination excluding, two lateral sprigs have grown, one of them was treated with exogenous gibberellic acid. Thus, two inflorescences, fertile and with induced male sterility, were obtained from the same sunflower plant.

Treatment with exogenous gibberellic acid

GA_3 (Sigma) solution was prepared by dissolving GA_3 in the minimal amount of ethanol 96%, and bringing it up to remaining volume in distilled water to make a final concentration of 0.005%. The treatment with GA_3 solution by plant spray was carried out at the developing inflorescence buds period. At this stage, prior to the opening of the inflorescence, male meiosis occurs in disc flower anthers (Anascenco, 1971). Non-GA_3 treated plants (control) were sprayed with distilled water. For assaying non-GA_3 treated plants (control) and GA_3 treated plants, 24 h post-treatment were used.

Phytohormones extraction. The plant materials were collected at various vegetative stages that were correlated with development and microsporogenesis. Phytohormones assays were performed on cotyledons, apex with true 2-3 leaves, inflorescences without bracts, and inflorescence flowers without parenchyma tissues of peduncles. Slicing was performed from radial to head to analyze the anthers at different developmental stages on the single inflorescence. Fresh plant material (about 10g) was harvested in the morning. The samples were homogenized and fixed in cold (-20^{o}C) 80% acetone (1:30 ratio) and extracted over-night at 3-5^{o}C during 24h. After a series of organic extractions and purifications the extracts were dried in vacuum at 40^{o}C. The residue was dissolved in 0.1 ml N,O-bis(trimethylsilyl)-acetamid with addition of 0.05 ml of trimethylclorosilan (1%) and then subjected to chromatography.

Chromatographic analysis. Quantitative analysis of phytohormones was performed using gas-liquid chromatographic method and indole-3-acetic acid and gibberellic acid (Sigma) as internal standards, as described previously by Cavell et al. (1967) with modifications (Duca et al., 1997).

The chromatograph FRACTOVAP 4200 equipped with a detector of flame ionization, line programs for temperature MOD 410, integrator MEGA SERIES SP 4270, rustproof column (2m x 4mm) with 5% SE-30 DMCS Cromoton W, 60/80 mesh (0.15-0.2 mm) was used for analysis with gas carrier N_2 at a flow of 25 ml/min. Air flow was maintained at 300 ml/min, while hydrogen flow was 25 ml/min. The injector temperature was 210^{0}C, the detector temperature was 210^{0}C.

The phytohormones were determined in the following temperature regime: after the injection, the temperature was maintained at 60^{0}C for 4 min, from then on the temperature rate increase was 12^{0}C/min until the temperature of 220^{0}C was achieved. This temperature was maintained until the end of the analysis. The phytohormone content was expressed in ng per gram of fresh weight (ng/g fwt).

Data are presented as means ± SE (standard errors) of three separate experiments (n = 6 for each experiment) and Student's *t* test ($P< 0.05$and $P<0.09$) was used to determine the statistical significance of differences between genotypes.

RESULTS AND DISCUSSION

Plant hormone metabolism and maintaining the levels of hormonal balance in appropriate temporal and spatial patterns influence the intensity, localization, structure and quality of all morphogenetic processes. The pathway of GA biosynthesis and catabolism and their physiological role has been investigated over many years by a variety of approaches, including the application of active GAs, chemical inhibitors of GA biosynthesis, and the analysis of mutants of plants such as maize, pea, and Arabidopsis (Kende and Zeevaart, 1997).

IAA and GA_3 are essential hormones that act synergetically on diverse developmental processes in plants (Ross and O'Neil, 2001); moreover, auxins stimulate the gibberellin biosynthesis (Symoons and Reid, 2002). Based on this information, quantitative analyses of the hormonal balance variation have been performed in some sunflower genotypes including hybrid F_1 and parents lines, during different ontogenesis stages. Our results have shown the quantitative variation of IAA and GA_3 levels depending on the plant tissues, development stages (Duca and Port, 2002) and environmental factors (Duca et al., 2003).

The most interesting data obtained were related to hormone amounts in different sunflower genotypes making up the CMS-Rf genetic system. Thus, hybrid F_1 was shown to contain the highest IAA amount versus RW637Rf, male fertility restorer line, which had the lowest hormone level. These features were found for apex, leaves and inflorescences (Table 1). Hormone levels in roots showed no significant quantitative variations between studied genotypes.

It is known that IAA induces DNA replication. The highest IAA level of F_1 associated with increased mitotic activity (Capatana, 2004) and with other morphological and physiological indices (Duca and Port, 2002) suggests a correlation between IAA amount and heterosis. It is also possible that the low IAA amount at homozygote RW637Rf line is the cause of the reduced height of these plants.

Table 1. IAA amount of different sunflower genotypes, ng/g fwt

Genotype	Plants number: *fertile* / *sterile*	Phenotype Genotype	Ontogenetic phases			
			The first pair of true leaves		Inflorescence bud developing	
			Roots	Apex	Leaves	Inflorescence
F_1	*76* / *0*	fertile S Rf	32.79 ± 0.12	70.98 ± 0.31	62.99 ± 0.64	81.64±0.29
MB 514 CMS	*2* / *58*	sterile S rfrf	30.47 ± 0.24	57.03 ± 0.05	60.15 ± 1.17	77.40±0.83
RW 637 Rf	*78* / *0*	fertile F RfRf	31.71 ± 013	50.41 ± 0.25	54.99 ± 4.70	61.05±1.86
LSD	*0.95*		*0.093*	*0.432*	*0.265*	*0.367*
	0.99		*0.140*	*0.654*	*0.401*	*0.556*

S – male sterile cytoplasm. containing mitochondrial *orfH522*; F – male fertile cytoplasm.

It is important to accentuate that the gibberellin level in all studied tissues and genotypes of sunflower was four-six fold less than IAA, as has been shown for maize (Polevoi, 1992). The highest GA_3 concentration was found in the male fertile genotypes, F_1 hybrid and the RW637Rf line that was distinguished by the increased biosynthesis during ontogenesis (Table 2). The intensity of phytohormones accumulation, expressed by harmonic mean, was also significantly higher for RW637Rf line than for F_1 and MB 514 CMS line (Duca, 1998).

Table 2. Gibberellin content at different sunflower genotypes, ng/g fwt.

Genotype	Plants number: *fertile* / *sterile*	Phenotype Genotype	Ontogenetic phases			
			The first pair of true leaves		Inflorescence bud developing	
			Roots	Apex	Leaves	Inflorescence
F_1	*76* / *0*	fertile S Rf	2.04 ± 0.05	16.94 ± 0.03	18.07 ± 0.21	14,3 ± 0,24
MB 514 CMS	*2* / *58*	sterile S rfrf	0.36 ± 0.03	11.82 ± 0.87	7.21 ± 0.63	9,50 ± 0,42
RW 637 Rf	*78* / *0*	fertile F RfRf	0.97 ± 0.04	17.40 ± 0.20	17.30 ± 1.03	17,40 ± 0,52
LSD	*0.95*		*1.868*	*0.493*	*1.050*	*0.709*
	0.99		*2.828*	*0.747*	*1.590*	*1.074*

S – male sterile cytoplasm. containing mitochondrial *orfH522*; F – male fertile cytoplasm.

The gibberellins level showed maximum values in roots and leaves of heterozygote plants and in apex and inflorescences of homozygote plants, but these differences were not statistically significant, because they are not reliable either for 0.95 nor for 0.99 probability levels.

Isogenic lines and phenocopies of sunflower are a good experimental genetic system for investigation of phytohormones interactions and their role in gene expression. Thus, the IAA level during the ontogenesis of three sunflower lines: MB514, MB514 CMS and MB514 treated with exogenous GA_3 showed lower values in the homozygote line with male sterility than its male fertile analogue, characterized by normal bisexual flowers with fertile pollen (Table 3). As a result of an exogenous hormonal treatment, the microsporogenesis was blocked, this phenomenon being associated with significant increases in IAA amount during the inflorescence buds' developing and active growth stages.

Also, it was found that the nucleic acid level (especially of RNA) and protein biosynthesis was increased (Duca, 1998; Duca and Savca, 1998). But by blossoming phase the auxin content and the above mentioned parameters decreased as their levels became lower than those found at CMS lines (Table 3). At this reproduction stage, CMS plants and those treated with gibberellins displayed abnormally developed anthers and lack of pollen.

Table 3. Auxin content of three isogenic sunflower lines, ng/g fwt.

Genotype	Plants number	Phenotype Genotype	Ontogenetic phases		
			Bud development	Active growth	Blossoming
			Apex Inflorescence	Apex Inflorescence	Apex Inflorescence
MB 514	*60*	fertile	60.57 ± 1.14	61.50 ± 0.92	49,53 ± 2,49
		F rfrf	75.80 ± 1.23	76.90 ± 2.26	85,40 ± 0,28
MB 514 CMS	*58*	sterile	58.53 ± 2.08	59.13 ± 1.16	48,00 ± 2,08
		S rfrf	73.20 ± 2.22	74.10 ± 1.02	85,00 ± 1,41
MB 514 +CA_3	*10*	sterile	60.77 ± 0.94	85.57 ± 1.28	45,50 ± 0,59
		F rfrf	75.57 ± 0.77	88.47 ± 3.21	83,80 ± 0,71
LSD	*0,95*		*0,120*	*0.526*	*0.138*
			0,064	*0.241*	*0.037*
	0,99		*0,182*	*0.797*	*0.210*
			0,097	*0.365*	*0.057*

S – male sterile cytoplasm, containing mitochondrial *orfH522*; F – male fertile cytoplasm

Our results have shown that the maximum GA_3 content was in apex and inflorescence tissues of MB 514 line, and also in plants exogenously treated with GA_3 in contrast to the lowest hormonal content ascertained at the cytoplasmic male sterile analogous MB514 CMS (Table 4).

Table 4. Gibberellins content in three sunflower isogenic lines, ng/g fwt

Genotype	Plants number	Phenotype Genotype	Ontogenetic phases		
			Bud development	Active growth	Blossoming
			Apex Inflorescence	Apex Inflorescence	Apex Inflorescence
MB 514	*60*	fertile	17.90 ± 0.14	18.13 ± 0.47	24.50 ± 0.14
		F rfrf	18.50 ± 0.33	16.26 ± 0.32	18.37 ± 1.18
MB 514 CMS	*58*	sterile	9.50 ± 0.42	12.7 ± 0.19	16.70 ± 0.45
		S rfrf	7.20 ± 0.57	6.70 ± 0.47	12.33 ± 0.43
MB 514 +CA_3	*10*	sterile	17.90 ± 0.09	20.43 ± 0.58	18.40 ± 0.19
		F rfrf	18.50 ± 0.33	16.80 ± 0.19	13.40 ± 0.52
LSD	*0.95*		*0.786*	*0.570*	*0.506*
			1.082	*1.051*	*0.573*
	0.99		*1.190*	*0.863*	*0.766*
			1.693	*1.591*	*0.868*

S – male sterile cytoplasm, containing mitochondrial *orfH522*; F – male fertile cytoplasm

During blossoming stage, the gibberellins quantity of GA_3 treated MB514 line decreased by approximately 30% in comparison to CMS analogue and by 20% compared to untreated MB 514. MB 514 CMS plants had low concentrations of this hormone compared to male fertile plants during all the ontogenetic phases studied. Gibberellins content boost at MB514 fertile line happened during ontogenesis stages, reaching higher levels at blossoming stage (24.5 ng/g fwt). Exogenous gibberellins application changed its internal concentration. Maximum values of endogen IAA and GA_3 content were determined at inflorescence development stage, 24 hours post treatment, and also during active growth phase.

Data on isogenic lines study provided us with more complete information related to auxin-gibberellin regulation of generative differentiation processes in sunflower. A comparative analysis of endogen auxins and gibberellins levels at different microsporogenesis stages (Fig. 1 and 2) revealed that phytohormone concentration decreased in disc flowers from the centre of the inflorescence to the periphery during the microsporogenesis in all studied genotypes.

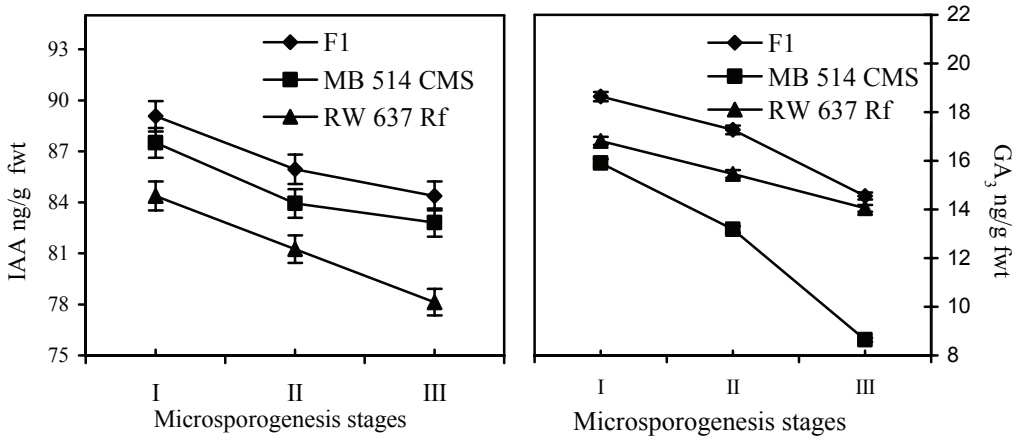

Fig. 1. Phytohormone levels in flowers at various microsporogenesis stages:
I – arhesporogenesis; II – sporogenesis; III - carpogenesis.

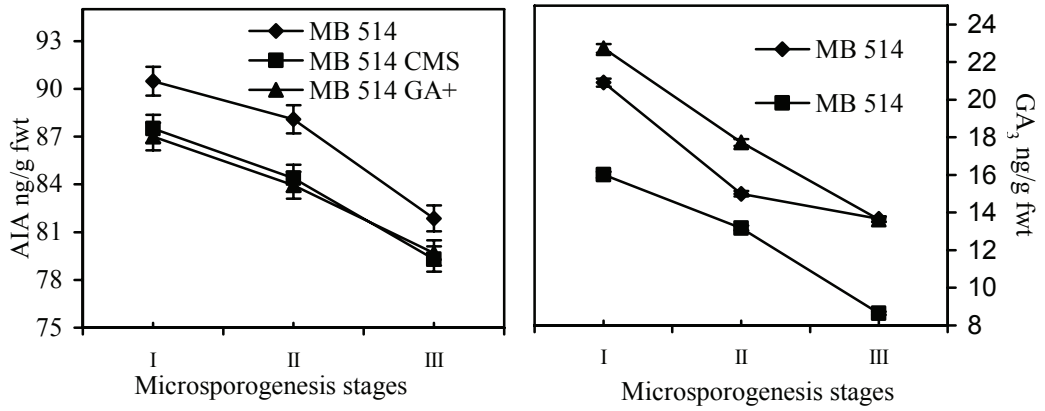

Fig. 2. Differences in phytohormone levels in flowers of three isogenic sunflower lines at following stages of microsporogenesis: I– arhesporogenesis; II – sporogenesis; III - carpogenesis

Our data support the findings related to the higher contents of both hormones in hybrid plants and the lowest IAA level in the male fertility restorer lines, but the lowest GA_3 level in the CMS plants.

Exogenous GA_3 treatment induced a decrease of the IAA concentration in disk flowers. The results obtained have shown no significant differences between plants with the male sterility induced and CMS plants. But the endogen gibberellin content in the treated genotype was higher than those detected in another two isogenic lines.

Besides the complex functional role of the studied parameters, the investigation of the IAA and GA_3 content in different sunflower genotypes during the ontogenesis has revealed several features regarding growth regulators levels and genetic CMS-Rf system. Thus, the outcomes showed a high level of GA_3 at RW 637 Rf line in comparison to other genotypes. MB 514 CMS line contained the lowest level of gibberellins, which increased during all the analyzed phases, even in disc flowers, where for RW 637 Rf line and F_1 a diminution in this hormone quantity was found. In fertile line MB 514 (as in other male fertile genotypes) there was a high auxin and gibberellin content during all studied phases in comparison to its male sterile analogue.

Genotypic peculiarities related to the auxin content were less considerable and less specific than those revealed by gibberellins content, which apparently verified their insignificant functional role in the phenotypic expression of CMS-Rf system. However, it could be supposed that a high gibberellin content is associated with restored male fertility, and a low auxin content with pollen sterility.

Evidence of the requirements of GAs in male reproductive development of flowering plants has resulted from genetic and physiologic studies of GA biosynthesis mutants. Typically, in addition to the dwarf stature, the GA-deficient mutants exhibit various defects of reproduction development (Kende and Zeevaart, 1997). Out of the majority of the plant growth regulators used as gametocydes (Frank et al., 1978), only gibberellins induce male sterility (Anascenco, 1971). These data together suggest that microsporogenesis development occurs normally at a sufficient level of GA. A low level of this hormone in the MB514 CMS line and a high level at RW 637 Rf line (and at all male fertile line) could support the hypothesis proposed. Also, these conclusions are sustained by the reported data that have shown that tomato sl_2 gene mutants (nuclear male sterility) contain a higher IAA and abscissic acid quantity but a lower gibberellin content (Santokh and Sowhneu, 1993).

Thus, it can be concluded that quantitative differences in auxins and gibberellins in various sunflower genotypes have revealed that self-regulation of the CMS-Rf system in sunflower is mediated by endogenous phytohormone concentration, depending on the genotype, ontogenesis phase and organ studied.

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