Theoretica and Applied Genetics 43, 27—30 (1973)

Mitomycin C Induced Leaf Mosaicism in *Glycine max* (L.) Merrill in Relation to the Post-Germination Age of the Seed

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Summary. The frequency of mitomycin C induced somatic crossing over in variety L65—1237 of *Glycine max* is shown to be dependent upon the (physiological) age of the seed during post germination period. Effect of mitomycin C during the first four hr of germination is significantly lower than during later periods. This increase in the frequency of somatic crossing over is observed up to about 20—24 hr and is then followed by a decrease. These changes did not appear to be related to the onset and pattern of synthesis of DNA or/and proteins in the embryonic tissues. However, mitomycin C is effective even when no DNA synthesis is going on.

Introduction

Variety L65-1237 of *Glycine max* (L.) Merrill (Soybean) exhibits incomplete dominance of the chlorophyll development gene Y_{11} over its allele y_{11} so that $Y_{11}Y_{11}$ plants are dark green in color, $Y_{11}y_{11}$ are light green and $y_{11}y_{11}$ have golden yellow leaves. The leaves of the heterozygotes are often seen with three types of spots which may be (1) dark green, resembling in color the leaves of $Y_{11}Y_{11}$ plants, (2) golden yellow like $y_{11}y_{11}$ leaves in color and (3) twin or double where a dark green spot is adjacent to a yellow spot of approximately similar shape and size. The leaves of $Y_{11}Y_{11}$ and $y_{11}y_{11}$ plants are devoid of any spots. It was postulated (Vig and Paddock, 1968) that the twin or double spots have their origin in the process of somatic crossing over and that the single spots generally result from the failure of one of the two components of the double spots. A few single spots may also have their origin in chromosomal disturbances observed during mitosis (Vig, 1969). The general absence of any kinds of spots on the leaves of the homozygotes rules out the possibility of point mutations in the origin of these spots.

The frequency of this phenomenon responsible for spotting has been increased with the use of mitomycin C (Vig and Paddock, 1968) and cold shocks (Vig and Paddock, 1970). Agents like ethyl-methane sulfonate and daunomycin which are known to cause chromosomal aberrations but do not cause somatic exchanges to occur at chromosomal level (Vig and Paddock, 1970) are not effective in inducing spot formation. However, during the conduct of these experiments, it was observed that mitomycin C given to germinating seeds is less effective during the first few hours of germination than during the later part. This report summarizes the results obtained by treating the seeds during different phases of germination and some inferences regarding the time and mode of action of mitomycin C.

Materials and Methods

One year old seeds of variety L65-1237 were used in this study. Solutions of the antibiotic mitomycin C (Nutritional Biochem., Cleveland, Ohio) were prepared fresh in distilled water just before treatment and applied at the rate of 25 cc per hundred seeds treated. Germinating seeds when not in contact with mitomycin C were kept in clean petri dishes in 20—25 cc of distilled water. At the completion of the treatment, the seeds were washed thoroughly in running tap water followed by rinsing with distilled water. The seeds were then sown in galvanized iron flats containing heat sterilized soil uniformly mixed to fill the flats to about 3/4th of their depth. Details for individual experiments are provided in the text of the following section. All experiments were conducted in the green house fumigated against insects and at somewhat controlled temperature (85—95 °F) and approximately 30% humidity. Spots were counted from the two simple leaves and first compound leaf. For sake of simplicity in analysis each compound leaf was considered equivalent to 3 simple leaves. Statistical analysis was performed by using *t*-test (on Sigma-7 computor).

For the study of DNA and protein synthesis in germinating seeds, samples were taken from the seeds soaked in water containing 2 μc/ml of TdR-^{3}H (for incorporation into DNA) and Arg-^{3}H (for incorporation into proteins). In the first experiment the seeds (5 seeds per sample) were thoroughly washed after treatment, seed coat and cotyledons removed and then the embryo crushed direct in the NCS reagent (Nuclear chicago) and prepared for count of disintegrations per minute using a scintillation counter (Packard tricarb, 3000 series). In the second experiment the embryos from 5 seeds (per sample) were removed, washed, separated into root and leaf portions and then prepared for scintillation counting. The data was transformed into graphs reading for post germination time v/s percentage of cumulative activity in relation to the maximum total activity observed in that experiment.

Experimental Results

The pilot experiments were conducted by treating the seeds with water followed by 0.0025% mitomycin C treatment during 0—4, 12—16 and 20—24 hr in experiment 1 (Table 1) and 0—4, 6—10, 18—22 and 24—28 hr in the follow-up experiment, number 2 (Table 2). It is undoubtedly clear that in both the cases there is an increase in spot frequency in the material treated with mitomycin C over the control (material not treated with mitomycin C). Also it is evident (Table 1) that the material treated during 0—4 hr has far lower frequency of any kind of spots than the data obtained from 12—16 hr or 20—24 hr

Table 1. *Data on the frequency and proportions of different types† of spots on the leaves of $Y_{11}y_{11}$ Glycine max var. L65-1237 treated with 0.0025% of Mitomycin C during different periods of germination (0 hour = time of initiation of soaking the dry seeds)*

Treatment Period* (in hr)	Number of leaves analyzed	Spot frequency and type per leaf				
		DG	Yl	Db	T	*t*-Test**
1. —(water only)	120	0.04	0.13	0.08	0.25	
2. 0—4	160	0.85	0.52	1.14	2.51	1
3. 12—16	80	1.04	1.37	2.15	4.56	1, 2
4. 20—24	135	1.36	1.50	2.82	5.68	1, 2, 3

† DG = dark green, Yl = yellow, Db = double or twin.

* Refers to the period during which seeds were in contact with mitomycin C solution. Seeds were kept in H_2O before and after this period for a total duration of 24 hr.

** Number refers to the preceding treatments from which this treatment differs significantly (5% level).

Table 2. *Frequencies and proportions of different types† of spots on the leaves of Glycine max var. L65-1237 treated with 0.0025% of mitomycin C during different periods of germination (0 hour = the time of initiation of soaking the seeds)*

Treatment Period* (in hr)	Number of leaves analyzed	Spot frequency per leaf				
		DG	Yl	Db	T	*t*-Test**
1. —(water)	135	0.10	0.19	0.22	0.51	
2. 0—4	135	0.54	0.50	0.80	1.84	1
3. 6—10	135	0.87	0.78	1.67	3.32	1, 2
4. 12—16	135	0.92	1.21	1.48	3.61	1, 2
5. 18—22	125	0.80	1.10	1.50	3.40	1, 2
6. 24—28	145	0.75	0.90	1.13	2.78	1

† DG = dark green, Yl = yellow, Db = double or twin and T = Total spots.

* Refers to the period during which the seeds were in contact with the mitomycin C solution.

** Number refers to the preceding treatments from which this treatment differs significantly (5% level).

treatment. Statistical treatment of the data using analysis of variance and t-test confirmed the differences to be significant. Similar results from 0—4 hr treatment in Table 2 compared with 6—10 hr or any following treatment duration express significant differences. However, all the treatments in this experiment do not have significantly higher number of spots than the treatment preceding immediately. As a matter of fact, a decline in the number of spots has been observed in the material treated during 24—28 hr.

One may observe the decline of spots frequency in the last treatment in Table 2. The repetition of these experiments extending the treatment periods to beyond 24 hr is desired. It is also to be noticed that in the two experiments reported, several periods of treatments are not covered e. g., in experiment 1 (Table 1) 4—8, 8—12, 16—20 hr of treatments are missing and it is likely that any of these areas also might show a dip in the frequency of spots.

To meet the above requirement two more experiments were conducted to cover the 4 hr periods

Table 3. *Data on the frequency and proportions of different types† of spots on the leaves of $Y_{11}y_{11}$ Glycine max var. L65-1237 treated with 0.0025% of mitomycin C during different periods of germination (0 hour = time of initiation of soaking the dry seeds)*

Treatment Period* (hr)	Number of leaves analyzed	Spot frequency and type per leaf				
		DG	Yl	Db	T	*t*-Test**
1. (0—36 hr H_2O)	125	0.07	0.09	0.05	0.21	
2. 0—4	120	0.11	0.22	0.28	0.61	1
3. 4—8	90	0.20	0.33	0.47	1.00	1
4. 8—12	125	0.46	0.93	0.92	2.31	1, 2
5. 12—16	120	0.38	0.43	0.61	1.42	1, 2
6. 16—20	100	0.32	0.62	0.64	1.58	1, 2
7. 20—24	75	0.53	0.89	1.63	3.05	1, 2
8. 24—28	100	1.24	1.49	2.06	4.79	1, 2, 3, 4, 5, 6
9. 28—32	85	1.45	2.06	2.76	6.27	1, 2, 3, 4, 5, 6, 7
10. 32—36	105	0.72	0.91	1.37	3.00	1, 2, 3, 5, 6, 9

† DG = dark green, Yl = yellow, Db = double or twin and T = total spots.

* Refers to the period during which seeds were in contact with mitomycin C solution after water soaking for the period indicated by the 1st hour of mitomycin C treatment.

** Number refers to the preceding treatment from which this treatment differs at 5% level of significance.

starting with 0 hr and ending at 36th hr (Tables 3 and 4). An analysis of Table 3 reveals as steady increase in the frequency of spots up to 28—30 hr treatment and then sudden drop for the material treated during the next 4 hr. The only reading not in line with the rest of the data is the frequency of spots in the material treated during 8—12 hr. The data in Table 4 is also similar in main characteristics

Table 4. *Data on the frequency and proportions of different types† of spots on the leaves of $Y_{11}y_{11}$ Glycine max var. L65-1237 treated with 0.0025% of mitomycin C during different periods of germination (0 = time of initiation of soaking the dry seeds)*

Treatment Period* (hr)	Number of leaves analyzed	Spot frequency and type per leaf				
		DG	Yl	Db	T	*t*-Test**
1. —	135	0.33	0.18	0.22	0.73	
2. 8 hr H_2O	85	0.19	0.29	0.19	0.67	
3. 0—4	80	0.35	0.36	0.33	1.04	1
4. 4—8	110	0.56	0.49	0.83	1.88	1, 2, 3
5. 8—12	140	0.67	0.95	1.41	3.04	1, 2, 3
6. 12—16	145	1.03	0.95	1.34	3.32	1, 2, 3
7. 16—20	105	0.64	0.50	0.78	1.92	1, 2
8. 20—24	140	1.51	1.51	1.72	4.76	1, 2, 3, 4, 7
9. 24—28	150	0.73	0.92	0.95	2.60	1, 2, 3
10. 28—32	115	0.78	1.35	1.30	3.43	1, 2, 3
11. 32—36	140	1.01	1.56	1.26	3.83	1, 2, 3, 4

† DG = dark green, Yl = yellow, Db = double or twin and T = total spots.

* Refers to the period during which seeds were in contact with mitomycin C solution. Seeds were kept in H_2O before and after this treatment for a total of 36 hours before sowing.

** Numbers refer to the treatments which differ significantly from the treatment in question (at 5% level).

Table 5. *Frequency and proportions of spots on the* $Y_{11}y_{11}$ *leaves analyzed in experiments 5 and 6. Mitomycin C (0.0025%) was used during different 4-hr intervals during the first 24 (expt. 5) or 32 (expt. 6) hr of germination*

	Treatment Period (hr)	No. of leaves analyzed	Type and frequency of spots per leaf			
			DG	Yl	Db	T
Experiment 5	1. 0—4	40	0.22	0.27	0.31	0.80
	2. 4—8	80	0.38	0.43	0.29	1.10
	3. 8—12	80	1.03	0.53	1.53	3.09
	4. 12—16	80	0.16	0.26	0.21	0.63
	5. 16—20	80	0.57	0.60	1.17	2.34
	6. 20—24	100	0.69	0.66	0.94	2.29
Experiment 6	1. 0—4	70	0.09	0.26	0.51	0.86
	2. 4—8	35	0.29	0.40	0.49	1.18
	3. 8—12	65	1.08	1.43	2.00	4.51
	4. 12—16	40	0.70	0.78	1.38	2.86
	5. 16—20	50	0.34	0.46	0.54	1.34
	6. 20—24	80	0.60	1.04	1.71	3.35
	7. 24—28	65	0.46	0.92	1.11	2.49
	8. 28—32	55	0.29	0.65	1.11	2.06

excepting that decline in spot frequency is observed to start rather early (compared to Table 3).

The observation of increase in spots related to the physiological advance of the soaked seeds is interesting. However, it is worth noticing that in both the above experiments what is called the experimental error may really be a part of the true cyclic increase/decrease in spot frequency. Two more experiments were carefully conducted to test the treatment up to 24 and 32 hr. Table 5 summarizing the results of the two experiments (5 and 6) offers further confirmation of a cyclic response of the seeds to mitomycin C sensitivity. First there is an increase in the frequency of spots up to about 12 hr, then a sudden decrease lasting another 4—8 hr and again a rather sharp increase. This is followed by a tendency of another decrease by 28th hr in experiment 6.

One more experiment was performed to complete a 24 hour treatment cycle. The seeds were treated with water, followed by 0.0025% mitomycin C solution and then with water again for the balance of 24 hr. Results (Table 6) are similar to the ones from the previous experiments (Tables 3, 4 and 5) in general details; i. e., the frequency of spots is increased by mitomycin C with an increase with the physiological age of the seed to an extent and then drops off to be followed by perhaps another increase.

Experimental results in relation to the study of DNA and protein synthesis in the germinating seeds have been reproduced in Figs. 1, 2 and 3*. The general pattern in all the cases is similar in that protein synthesis starts much earlier than synthesis of DNA and shows ups and downs in early stages of germination. Such does not appear to be true of DNA synthetic patterns which initiate late, show a sharp rise then a plateau then another sharp increase. Since the proteins studied in this case are the total

* The curves show cumulative incorporation of TdR-^{3}H or Arg-^{3}H.

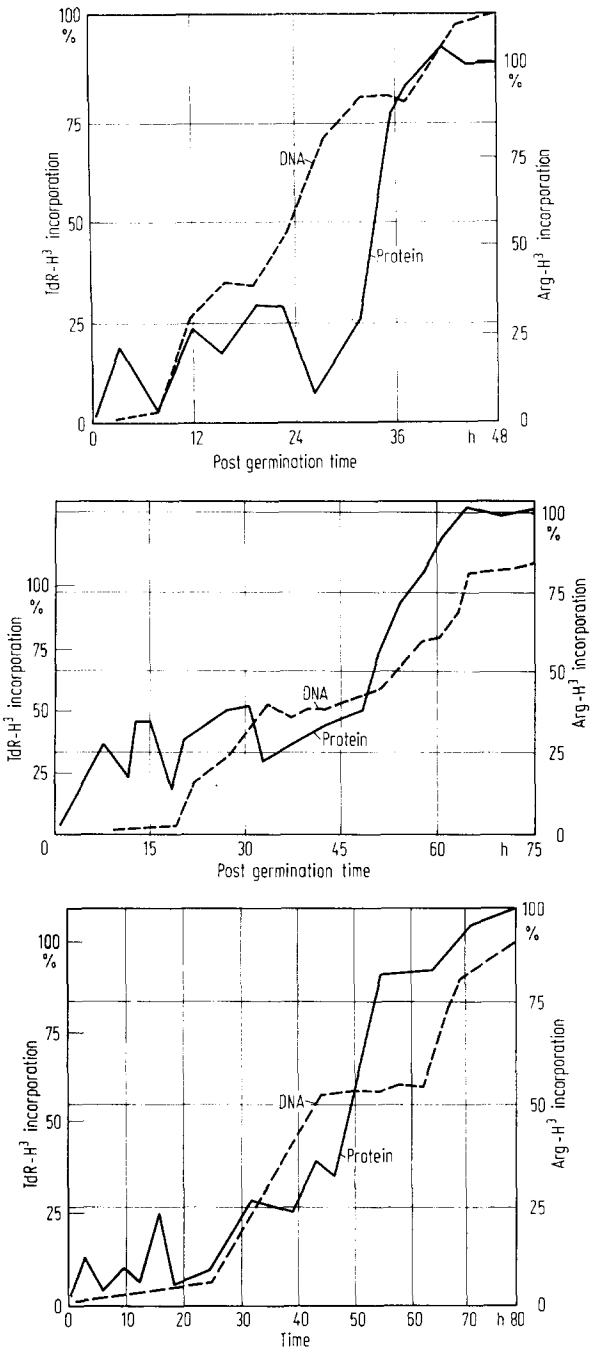

Fig. 1—3. Patterns of DNA and protein synthesis as revealed by TdR-^{3}H and Arg-^{3}H incorporation, respectively. The graphs show cumulative radioactivity in relation to the maximum calculated at the end of the experiment. Notice the uniform patterns of DNA synthesis in all the three studies. The interpretation of the data for early stages of protein synthesis appears impossible unless some fractions of proteins synthesized disintegrate quickly to release the radioactivity into the medium or moves into the cotyledons or unless there is constant experimental error in all the three experiments. Fig. 1. Study of whole embryos up to 48 hr, Fig. 2. Study of only the root part of the embryos during 75 hr, and Fig. 3. Study of only the leaf structures and stem apex. In each case 5 seeds per sample were used. Dotted line — DNA synthesis, solid line — protein synthesis.

Table 6. *Data on the frequency and proportions of different types† of spots on the leaves of* $Y_{11}y_{11}$ *Glycine max var. L65-1237 treated with 0.0025% of mitomycin C during different periods of germination (0 hour = time of initiation of soaking the dry seeds)*

Treatment Period* (hr)			Number of leaves analyzed	Spot frequency and type per leaf			
				DG	Yl	Db	T
H_2O	MC	H_2O					
1. —	—	24	85	0.13	0.19	0.16	0.48
2. 0	4	20	75	0.37	0.27	0.59	1.23
3. 4	4	16	50	0.32	0.50	1.08	1.90
4. 8	4	12	75	0.31	0.49	0.52	1.32
5. 12	4	8	60	0.52	0.45	1.22	2.19
6. 16	4	4	80	0.66	0.74	1.49	2.89
7. 20	4	—	80	0.39	0.49	1.18	2.06

† DG = dark green, Yl = yellow, Db = double or twin and T = total spots.

* Refers to the period during which seeds were in contact with mitomycin C solution and/or water in the sequence given.

cellular proteins and not necessarily the chromosomal proteins it is difficult to relate the changes in the frequency of spots to either component of cell. The results regarding DNA synthesis agree with earlier interpretation of Miksche's data (Miksche, 1966) that one wave of DNA synthesis is completed by about 30—35 hr and then another starts by about 48th hr or so. The pattern of spot frequency does not appear to correspond with this pattern of synthesis. As a matter of fact a declining trend in spot frequency is observed by the time the 1st wave of DNA synthesis is about midway. The curves about protein synthesis could not be interpreted intelligently in view of the peaks and declines during the early hours of Arg-^{3}H incorporation. But since this trend was observed in all the three experiments, it was considered worthwhile to present the data as such.

Discussion

Several suggestions can be made to accommodate the increased effectiveness of mitomycin C with an increase in the physiological age of the seed. The first to come to mind is the differential permeability of the cell wall to the mitomycin C molecules so that the amount of the antibiotic to enter the seeds (cells) is less during early stages of germination than during the later stages. Thus, it becomes a question of differential availability rather than differential effectiveness of the drug. That such is not the case is demonstrated by the apparent cyclic behavior of cells as related to the frequency of spots. Thus, decrease in spot frequency during the middle of treatment period in several experiments is certainly not entirely due to the low permeability of the cell wall or low water contents of the seed, or consistant experimental errors.

Cyclic increase/decrease in spot frequency as observed in the foregoing experiments cannot be correlated with the onset of synthesis or quantities of either DNA or proteins in the embryonic cells with any certainty. The pattern of DNA synthesis and mitotic activity in the embryonic cells in this study as well as those by Miksche (1965, 1966) carried out on the quiescent zones in roots during early embryonic stages fail to show any relationship either between DNA synthesis or mitotic activity and the spot frequency.

The synthesis of proteins in the embryo during initial stages of germination also fail to establish any definite relationship with the change in frequencies observed. However, mitomycin C is effective even at stages when DNA synthesis can hardly be conceived to begin, e. g., the first 0—4 hr. The data also fail to establish if any respectable quantity of mitomycin C binds to DNA and thus persists in these early cells to become effective at a later time because then one would expect a plateau of the curve representing spot frequency once it reaches a certain level.

One wonders if synthesis of proteins of any kind or some sort of enzyme(s) are responsible for the results observed. It is now known that protein synthesis in general starts in the cell much earlier than the synthesis of DNA. Perhaps the differential response of mitomycin C is because of synthesis of one or a few protein fractions depending upon different stages of cell cycle and relative frequencies of cell populations in different sub-phases (G_1, S, G_2) of interphase. No conclusive suggestion can, however, be made at this time.

Acknowledgement

I wish to express my thanks to the Research Advisory Board of the University of Nevada for financial aid, Dr. Cliff Blincoe of the Department of Biochemistry of this campus for helping with Scintillation counter work and Dr. R. L. Bernard, USDA, Urbana, Illinois, for the supply of original seed of L65-1237.

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Received March 22, 1972

Communicated by H. Stubbe

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