

Embryo culture of *Medicago scutellata* and *M. sativa**

G.R. BAUCHAN

U.S. Department of Agriculture, Agriculture Research Service, Building 001, Room 311,
BARC-West, Beltsville, MD 20705, USA

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Abstract. Resistance to the alfalfa weevil (*Hypera postica* (Gyllenhal)) and the potato leafhopper (*Empoasca fabae* (Harris)) is lacking in cultivated alfalfa. However, a closely related annual *Medicago*, *Medicago scutellata*, possesses dense glandular stem and leaf hairs which provides a mechanism for resistance. Several attempts have been made at transferring the glandular haired trait from *M. scutellata* to perennial alfalfa with limited success. Earlier studies have shown that one reason for the lack of success is embryo abortion. Therefore, this study was initiated to observe zygotic embryo-genesis and to develop an embryo rescue technique for *M. scutellata* and *M. sativa*. Observations of zygotic embryogenesis showed that the two species are similar in morphology and can be described from youngest to oldest as globular, heart, torpedo, and hook shaped embryos. *M. sativa* embryos are smaller than *M. scutellata* embryos and develop three to four days later. Self pollinated *M. scutellata* (PI 307446) and sib mated *M. sativa* (Saranac AR) embryos were cultivated on Murashige and Skoog (MS) basal medium with various combination of 2,4-dichlorophenoxyacetic acid (2,4-D), indolacetic acid (IAA), 6-benzylaminopurine (BAP), and kinetic (KIN). Embryos from both species were also cultured on Schenk and Hildebrandt's (SH) basal medium with the addition of L-glutamine and L-proline. The experimental design was a completely randomized factorial for each experiment. Heart and torpedo shaped embryos from *M. scutellata* grew best (27.5% plantlet recovery) when cultured on MS medium with 0.05 mg l^{-1} of both IAA and BAP. After 15 to 30 days on this medium, the embryos had only developed shoots. Therefore, it was necessary to transfer the shoots to MS basal medium without phytohormones for rooting. Rooting occurred in 15 to 30 days and the plantlets could be acclimatized to soil within 2 to 4 weeks. *M. sativa* embryos grew best (31% plantlet recovery) on SH medium with 50 mM L-glutamine. *M. sativa* embryos developed both shoots and roots on this medium. This information may now be applied to the development of an embryo culture method for recovering insect resistant hybrids between *M. scutellata* and *M. sativa*.

Introduction

There is no known variety of cultivated alfalfa, *Medicago sativa* L., that is highly resistant to either the alfalfa weevil, *Hypera postica* (Gyllenhal), or

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the potato leafhopper, *Empoasca fabae* (Harris). However, some related species, including *M. disciformis* DC., *M. minima* Bart., *M. polymorpha* L., *M. rugosa* Desr., *M. scutellata* Mill., and *N. truncatula* Gaertn. [2, 23, 24] possess dense glandular stem and leaf hairs as a mechanism for insect resistance [24].

Several attempts have been made at transferring the glandular hair trait through interspecific hybridization [6, 7, 15]. Sangduen and co-workers [20] studied the pollen tube growth of *M. scutellata* in male sterile *M. sativa* styles and noted that few pollen grains penetrated the style. Abnormal pollen tube-style interactions occurred and fertilization was very infrequent. Light and electron microscope studies of the developing embryo showed that the maternal and embryonic tissues failed to break down and transport nutrients, thus the developing embryos did not mature [21].

Despite these barriers, Sangduena and co-workers [19] were the first to report the production of a hybrid between *M. sativa* and *M. scutellata*. The hybrid was obtained by applying gibberellic acid to the peduncle and pedicels of *M. sativa* immediately after pollination with pollen from *M. scutellata*. This procedure allowed the developing pod to remain on the plant until a mature seed was formed. The hybrid plant was perennial, possessed no glandular hairs, was intermediate for some morphological characteristics, and was both male and female sterile [19].

Interspecific hybridization between perennial *Medicago* species using ovule culture followed by embryo culture has been successful in recovering hybrid embryos which would not mature in vivo [11, 12]. Interspecific hybrid embryo culture has been successful in other Leguminosae species such as *Arachis* [1], *Glycine* [14], *Lathyrus* [16], Lotus [9], *Melilotus* [26], *Ornithopus* [27], *Trifolium* [18] and *Vigna* [8].

Transfer of the glandular hair trait from annual *M. scutellata* to alfalfa via interspecific hybridization may require the use of an embryo culture system. The purpose of this study were to observe embryogenesis of zygotic embryos from *M. scutellata* and *M. sativa*, and to develop techniques and media for the development of immature embryos in vitro of *M. scutellata* and *M. sativa* in order to provide baseline data for a study of the rescue of hybrid embryos.

Materials and methods

Embryo development studies were conducted on *M. scutellata*, PI 307446, and sib-mated *M. sativa* Saranac AR. The plants were grown in a growth chamber with a 16 h day photoperiod maintained with cool white fluores-

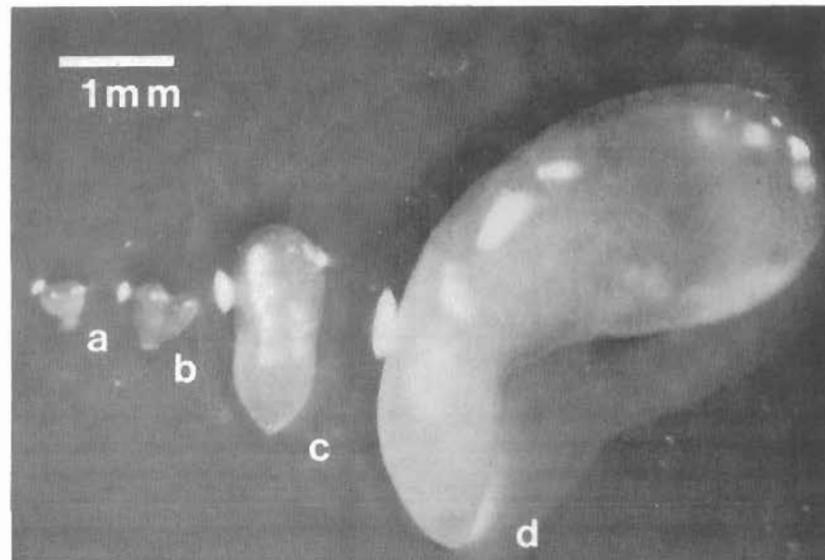


Fig. 1. Development of *Medicago scutellata* zygotic embryos. (a) globular shaped embryos (2-4 DAP); (b) heart shaped embryos (4-8 DAP); (c) torpedo shaped embryos (8-12 DAP); and (d) hook shaped embryos (12-16 DAP).

cent tubes (Philips F96T12-CW) providing a photosynthetic photon flux (PPF) of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (400 to 700 nm). Temperatures ranged from 24° and 28°C and the relative humidity was above 70%.

Immature pods were collected 2 to 20 days after pollination (DAP). Pods were surface sterilized briefly in 70% ethanol, soaked for 15 min in 0.5% sodium hypochlorite, and rinsed three times in sterile distilled water. Ovules were dissected and placed in a drop of sterile water under a dissecting microscope. Embryos were dissected from the ovules and classified as globular, heart, torpedo, or hook (Fig. 1, youngest to oldest embryo) and cultured on Murashige and Skoog's (MS) complete basal medium [13] supplemented with 30 g l^{-1} sucrose and varying amounts of phytohormones (Table 1, 2, 3). Schenk and Hildebrandt's (SH) complete basal medium [22] was also used with the same amounts of sucrose and agar as in the MS media. Additions to the SH medium were L-glutamine alone or in combination with 50 mM L-proline (Table 4). All amino acids were filter sterilized (Millipore $0.22 \mu\text{M}$) and added to cooled autoclaved basal medium. All media were adjusted to pH 5.8 before autoclaving at 121°C for 15 min and solidified with 10 g l^{-1} Difco Bacto agar. Five embryos per dish were placed on 12 ml of media per 60 mm petri dish. Petri dishes (experimental unit) were sealed with parafilm and placed on a culture shelf. All experiments were

Table 1. Number of *Medicago scutellata* embryos^a which developed into plantlets in response to different concentrations of IAA and KIN in MS basal medium and the analysis of variance of the treatment effect.

Conc. IAA mg l ⁻¹	Conc. KIN mg l ⁻¹				
	0.0	0.01	0.05	0.1	0.5
0.0	2	2	2	0	3
0.01	0	5	3	0	2
0.05	1	2	1	4	4
0.1	5	1	4	5	5
0.5	1	2	4	7	5

^a Each treatment consisted of 40 embryos, 20 heart and 20 torpedo stage embryos.

Source	DF	Mean square ^b	F value	P
IAA (I)	4	0.10	2.13	0.079
KIN (K)	4	0.02	0.49	0.740
I × K	16	0.06	1.27	0.224
Error	175	0.05		
CV%		19.44		
R ² %		0.15		

^b Analysis was performed on the square root of the number of plantlets

Table 2. Number of *Medicago scutellata* embryos^a which developed into plantlets in response to different concentrations of 2,4-D and BAP in MS basal medium and the analysis of variance of the treatment effects.

Conc. 2,4-D mg l ⁻¹	Conc. BAP mg l ⁻¹					
	0.0	0.001	0.01	0.05	0.1	0.5
0.0	1	1	0	1	0	0
0.001	0	0	3	4	3	0
0.01	1	0	0	1	1	0
0.05	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.5	0	0	0	0	0	0

^a Each treatment consisted of 20 embryos, 10 heart and 10 torpedo stage embryos.

Source	DF	Mean square ^b	F value	P
2, 4 D (D)	5	0.12	8.48	0.0001
BAP (B)	5	0.05	3.52	0.0055
DXB	25	0.04	3.14	0.0001
Error	108	0.14		
CV%		11.23		
R ² %		0.56		

^b Analysis was performed on the square root of the number of plantlets.

Table 3. Number of *Medicago scutellata* embryos^a which developed into plantlets in response to different concentrations of IAA and BAP in MS basal medium and the analysis of variance of the treatment effect.

Conc. IAA mg l ⁻¹	Conc. BAP mg l ⁻¹				
	0.0	0.01	0.05	0.1	0.5
0.0	2	3	3	3	0
0.01	0	5	1	1	0
0.05	1	0	11	6	0
0.1	5	3	1	5	0
0.5	1	2	2	2	0

^a Each treatment consisted of 40 embryos, 20 heart and 20 torpedo stage embryos.

Source	DF	Mean square ^b	F value	P
IAA (I)	4	0.07	1.45	0.2202
BAP (B)	4	0.30	6.00	0.0002
I × B	16	0.10	1.94	0.0199
Error	175	0.05		
CV%		20.19		
R ² %		0.26		

^b Analysis was performed on the square root of the number of plantlets.

completely randomized factorials with the 2 additions as factors in a completely randomized design with 5 replications for the hormone combination of indoleacetic acid (IAA) and Kinetin (KIN) (experiment 1); 6 replications for the hormone combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) (experiment 2); 5 replications for the hormone combination of IAA and BAP (experiment 3); and 2 replications for the amino acid combinations of L-proline and L-glutamine (experiment 4). The cultures were incubated at 24 to 28 °C under cool white fluorescent tubes (Philips F40CW) providing a PPF of 65 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (400 to 700 nm) and a 16 h photoperiod. Once the optimum medium was determined, approximately 100 embryos were re-tested to determine if the results were repeatable.

Shoots that developed which did not form roots were transferred, when they were one to two cm in length, to 50 ml screw cap bottles containing MS basal medium without phytohormones. When rooting had occurred (two to four weeks) plantlets were removed from the bottles, placed in peat pots filled with sterile soil, and covered with an inverted glass beaker for two weeks to protect them from desiccation.

The total number of plantlets per petri dish were counted. An analysis of variance was performed for each experiment. Data were transformed using the formula $(x + 1)^{1/2}$ to stabilize the variance.

Table 4. Number of *Medicago sativa* embryos^a which developed into plantlets in response to different concentrations of L-proline (P) and L-glutamine (G) in SH basal medium and the analysis of variance of the treatment effect.

Conc. P mM	Conc. G mM		
	0	25	50
0	0	14	31
50	0	3	2

^a Each treatment consisted of 100 embryos, 50 heart and 50 torpedo stage embryos.

Source	DF	Mean square ^b	F value	P
Proline (P)	1	1.43	17.28	0.0001
Glutamine (G)	2	0.77	9.33	0.0002
P × G	2	0.57	6.86	0.0015
Error	114	0.08		
CV%		25.16		
R ² %		0.30		

^b Analysis was performed on the square root of the number of plantlets.

Results and discussion

Zygotic embryogenesis was first described in alfalfa by Cooper [5]. This study confirms Coopers light microscopic observations of embryo development. The two species were similar, although, *M. sativa* embryos were smaller than *M. scutellata* (ie. hook stage embryos from *M. scutellata* are 4 mm, whereas *M. sativa* are 2.5 mm in length) and the zygotic embryos of *M. scutellata* developed much faster than those of *M. sativa*. Globular stage embryos (Fig. 1a) could be seen at 2 to 4 DAP in *M. scutellata*, whereas globular stage embryos of *M. sativa* did not appear until 4 to 8 DAP. Heart shaped embryos (Fig. 1b) of *M. scutellata* and *M. sativa* appeared at 4 to 8 DAP, and 8 to 12 DAP, respectively. Embryos resembling torpedos (Fig. 1c) developed in *M. scutellata* and *M. sativa*, 8 to 12 and 12 to 16 DAP. Further development of the cotyledons within the cramped seed caused the embryos to form a hook shape (Fig. 1d). These embryos appear at 12 to 16 DAP in *M. scutellata* and 16 to 20 DAP in *M. sativa*.

Several different tissue culture media, Linsmaier and Skoog [10], Blaydes [4], Philips and Collins [17], Murashige and Skoog [13], and Schenk and Hildebrandt [22], with various levels and combinations of 2,4-D, IAA, BAP, and KIN have been used by the author to culture embryos from both *M. scutellata* and *M. sativa* all with limited success. The media described in this study are the only media evaluated so far which allow normal embryo development into mature plants.

Experiment 1 indicated that IAA, KIN, and the interaction of the two hormones had no effect on *M. scutellata* plantlet recovery (Table 1). The hormone combination of 0.5 mg l^{-1} IAA and 0.1 mg l^{-1} KIN, however, gave the highest number of plantlets recovered for this experiment (Table 1). The combination of 2,4-D and Kin in a similar experiment produced no plantlets.

The analysis of variance for experiment 2 indicated that 2,4-D, BAP and their interaction was significant (Table 2). The best media for the recovery of *M. scutellata* plantlets for this experiment was 0.001 mg l^{-1} 2,4-D and 0.05 mg l^{-1} BAP (Table 2). Subsequently culturing of 95 heart stage and 104 torpedo stage embryos on this medium resulted in 13% heart stage embryos and 25% torpedo stage embryos developing into mature plants.

The third experiment's analysis of variance indicated that BAP and the interaction of IAA and BAP had significant effects on *M. scutellata* plantlet recovery (Table 3). The optimum medium for the culturing of *M. scutellata* embryos was MS media containing 0.05 mg l^{-1} IAA and 0.05 mg l^{-1} BAP (MS1) (Table 3). After further culturing of embryos on MS1, 6 of 50 heart stage embryos and 24 of 60 torpedo stage embryos developed into mature plants.

Shoots developed from the embryos of *M. scutellata* in 15 to 30 days after culturing on MS media with phytohormones. An additional 15 to 30 days on MS medium without phytohormones was required for rooting to occur. Eighty-five percent of the shoots that developed formed roots in 4 to 6 weeks. Acclimatization from the culture bottles to soil occurred within 2 to 4 weeks. Eighty-five percent of the plantlets which rooted in vitro survived acclimatization and flowered in the greenhouse.

Embryos from *M. sativa* did not develop on any media which contained phytohormones. The embryos usually turned yellow within 2 days and died within 6 days after culturing. Occasionally callus would form, however, no shoots ever appeared (author, unpublished). Therefore, SH medium supplemented with L-glutamine and/or L-proline were tested as the addition of these amino acids allowed the development of alfalfa somatic embryos in vitro [25].

The analysis of variance for experiment 4 indicated that the treatments and the combination of L-glutamine and L-proline had significant effects on the recovery of *M. sativa* embryo (Table 8). The optimum medium for the culturing of *M. sativa* embryos was SH medium with the addition of 50 mM L-glutamine (Table 4). L-proline had a negative affect on the recovery of plantlets. Subsequently culturing of embryos on SH medium plus 50 mM L-glutamine yielded 9 plantlets from 50 heart stage embryos and 20 plantlets from 50 torpedo stage embryos. *M. sativa* embryos developed shoots and

roots within 4 weeks after culturing in all of the SH media tested. *M. scutellata* embryos either produced callus or died on all of the SH media tested.

This study showed that the zygotic embryos of *M. scutellata* were larger and developed earlier during embryogenesis than *M. sativa*. It also indicated that immature heart and torpedo stage embryos from both *M. scutellata* and *M. sativa* can be placed into an artificial medium which will allow the embryos to develop into mature plants. This information can now be applied to the development of an embryo culture method for recovering hybrids between *M. scutellata* and *M. sativa*.

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