A New Method to Prepare Root Tip Chromosomes in Alfalfa¹

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Genetic and breeding investigations often require karyotype analysis, chromosome counting and banding. The precision and reliability of these investigations depend on the satisfactory preparation of metaphase cells with well-spread chromosomes. An improved chromosome preparation technique is needed for alfalfa *Medicago sativa* L. Most researchers have used similar procedures for alfalfa chromosome preparations (Lesins and Lesins 1979, Yen and Murphy 1979, Sangduen 1982, McCoy and Smith 1983, Wang, 1983, Baughan and Elgin 1984, Johnson 1984). With these procedures, it is difficult to obtain a monolayer of single cells on the slide. The bulk tissues resist the pressures of squashing and tapping and, thus, make it difficult to spread the chromosomes within the few cells which are separated from the bulk tissues. Enzyme treatment appears to be a promising way to separate groups of cells and to clear the cytoplasm. The purposes of our experiments were to test the effects of enzymes on slide preparations and to develop a satisfactory and efficient procedure for alfalfa chromosome analysis.

Materials and methods

We used root tips of: 1) *Medicago disciformis* DC. ($2n=2x=16$), a diploid annual species, 2) Lahontan, a cultivar of the tetraploid ($2n=4x=32$) perennial *M. sativa*, and 3) plants of Lahontan ($2n=4x=32$) regenerated from anther calluses. Enzyme preparations were: 1) 5% pectinase solution, 2) 5% cellulase solution, 3) a combination of 2.5% pectinase and 2.5% cellulase, 4) a combination of 5% pectinase and 2.5% cellulase and 5) a combination of 5% pectinase and 5% cellulase. Pectinase was the product of the Sigma Chemical Company. It is purified from *Aspergillus niger* and contains 0.9 units per mg of solid. Cellulase was the product of Yakult Honsha Co. Ltd. Durations of the enzyme treatments were 30, 45, 60, 75, 90 and 120 minutes at a temperature of 37°C. A duration of 5 hours was included for treatments 1 and 2.

Alfalfa seeds were sterilized in 30% Clorox bleaching solution (1.75% ai) with a constant stir for 3 minutes. Seeds of *M. disciformis* were scarified before being sterilized. Sterilized seeds were washed with tap water five or six times and placed on moistened filter paper in Petri dishes for germination. Seedlings with roots about 1.5 cm long were submerged for 2 hours in a saturated solution composed of equal parts of paradichlorobenzene and bromonaphthalene. Aeration was provided by pumping air into this solution. Roots were then cut from the seedlings and fixed for 1 hour in a solution of acetic acid and ethanol (1:1). The fixed roots were washed with distilled water 3 to 4 times before the enzyme solution (5 roots/ml) was added.

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After the enzyme treatment in a water bath at 37°C for the previously specified time intervals, the digested roots were removed from the the solution. The root tips were placed on dry slides, where they adhered to the glass surface. The remainder of the root was removed from the slide. One drop of 1% acetocarmine was added to the root tip immediately and the fine tip of the forceps was used to very gently tap (no more than 5 to 6 times) the root tip in the staining solution. The cells were thus separated and dispersed in the staining solution. After 1 to 2 minutes, a cover slip was placed over the cells within the staining solution. Excess staining solution was removed with Kimwipe paper, after which heavy pressure was applied to the cover glass. The slide was heated over an alcohol burner for several short periods to clear the background. The slides were temporarily sealed with Cover Glass Cement.

Table 1. Effect of enzyme solutions and duration of treatment on preparation of *Medicago* root tips for chromosome counts

Enzyme solution ¹	Duration of treatment (min)	<i>M. sativa</i> 'Lahontan'			<i>M. disciformis</i>			Regenerated Lahontan		
		No. of slides having CCC ²	Reliability (%)	No. of CCC per useful slide	No. of slides having CCC	reliability (%)	No. of CCC per useful slide	No. of slides having CCC	reliability (%)	No. of CCC per useful slide
1	30	0	0	0	1	25	5.00	0	0	0
	45	1	25	4.00	0	0	0	1	50	5.0
	60	3	75	4.33	1	25	7.00	1	50	7.0
	75	2	50	7.50	4	100	5.00	1	50	6.0
	90	3	75	6.67	3	75	5.67	2	100	5.5
	120	2	50	5.50	2	50	6.00	1	50	3.0
2	30	0	0	0	0	0	0	0	0	0
	45	0	0	0	0	0	0	0	0	0
	60	0	0	0	0	0	0	0	0	0
	75	0	0	0	0	0	0	0	0	0
	90	0	0	0	0	0	0	0	0	0
	120	0	0	0	0	0	0	0	0	0
3	30	1	25	5.00	2	50	5.00	0	0	0
	45	2	50	6.50	1	25	5.00	1	50	5.0
	60	3	75	6.33	3	70	6.00	2	100	5.0
	75	3	75	7.66	2	50	5.50	1	50	8.0
	90	4	100	6.25	3	75	7.66	2	100	5.5
	120	1	25	9.00	2	50	4.50	0	0	0
4	30	1	25	4.00	1	25	7.00	1	50	6.0
	45	1	25	11.00	1	25	5.00	0	0	0
	60	3	75	5.33	3	75	5.33	1	50	7.0
	75	3	75	8.33	4	100	6.75	2	100	6.5
	90	3	75	7.66	3	75	7.00	1	50	9.0
	120	1	25	6.00	2	50	4.50	1	50	5.0
Control 1		1	25	2.00	1	25	2.00	0	0	0
Control 2		1	25	2.00	2	50	1.00	1	50	1.0

¹ 1=5% pectinase, 2=5% cellulase, 3=2.5% pectinase+2.5% cellulase, 4=5% pectinase+2.5% cellulase.

² CCC=chromosome countable cells.

Control procedure #1 was as follows: 1) Roots were immersed in ice water for 24 hours, 2) fixed 3 to 4 days in a solution of ethanol and propionic acid (3: 1) containing a small amount of FeCl₃, 3) hydrolyzed in 1N HCl at 60°C for 6 minutes, 4) stained in 1% acetocarmine for several days, and 5) squashed. Control procedure #2 was the same as the enzyme treatments, except hydrolyzation in 1N HCl at 60°C for 6 minutes replaced the enzyme treatment. Four

slides were made of each treatment, except from roots of plants regenerated from calluses (2 slides).

The numbers of slides having chromosome countable cells (CCC) and the average number of CCC per useful slide (that had CCC) were recorded and reliability was calculated based on microscope observations.

Figs. 1–2. 1, photomicrograph showing the effect of enzyme treatment on staining and dispersion of *Medicago sativa* root tip chromosomes. $\times 1000$. 2, photomicrograph showing the effect of enzyme treatment on staining and dispersion of *Medicago disciformis* root tip chromosomes. $\times 1000$.

Since a combination of 5% pectinase and 2.5% cellulase proved to be the best enzyme preparation, it was used in additional experiments (using the above procedures except as noted) to determine if the technique could be improved. Experiment 1—Roots were pretreated in saturated solution of: 1) Paradichlorobenzene (12 hours) and 2) equal parts of paradichlorobenzene and bromonaphthalene (8 hours). Experiment 2—Roots were pretreated in saturated solutions of: 1) Paradichlorobenzene, 2) bromonaphthalene and 3) equal parts of 1 and 2. In each of these two experiments the roots were digested 90 minutes. Experiment 3—Roots were fixed in Carnoy's II fixation solution (ethanol:chloroform:acetic acid=6:3:1) for 1 hour. Root tips were digested 75 minutes.

Results

Enzyme treatments The roots from the annual diploid *Medicago disciformis*, the perennial tetraploid *Medicago sativa* 'Lahontan' and the plants regenerated from callus responded similarly to the kinds of enzymes and duration of treatments (Table 1). Enzyme treatment 4 (5% pectinase, 2.5% cellulase) for a duration of 60 to 90 minutes gave the best results. Its reliability and the number of chromosome countable cells were high. Distribution of cells and spreading of chromosomes on a single plane in the cells were easily achieved (Figs. 1, 2). Treatment 3 was equal to Treatment 4, except the roots were difficult to manipulate. The root tips became detached from the root proper and, consequently, were difficult to retrieve from the solution. Pectinase alone (Treatment 1) failed to completely digest the roots, even after treatment for 5 hours. The remaining tissue interfered with spreading the cells and placing chromosomes on a single focal plane in the cell. Also, semi-permanent slides gradually dark-

ened. Cellulase alone (Treatment 2) gave unsatisfactory results, i.e., it failed to adequately digest the root tips. They were still intact after treatment for 5 hours. A treatment of 5% pectinase and 5% cellulase was so drastic that controlling the treatment time became very critical and difficult.

An enzyme treatment duration of 60 to 90 minutes was most reliable for obtaining a high frequency of chromosome-countable cells. Shorter time periods resulted in incomplete digestion. If digestion time exceeded 90 minutes, many cells were too fragile to be processed. Although the root tips appeared to be intact, some cells were lost.

Both control treatments were inferior to enzyme Treatments 1, 3, and 4. A 6-minute hydrolysis in 1N HCl was insufficient to adequately separate cells and clear the background. The cells, however, were more tolerant to tapping than those treated with enzymes.

Pretreatment A saturated solution of paradichlorobenzene, bromonaphthalene, or a solution composed of equal parts of the two were equally satisfactory as a pretreatment to prepare roots for enzyme digestion (Table 2). A pretreatment of about 2 hours appeared to be the optimum time. Without pretreatment, good metaphase cells were infrequent.

Table 2. Effects of pretreatment of *Medicago sativa* 'Lahontan' on preparation of root tips for chromosome counts

Pretreatment of roots (2 hr)	No. of slides observed	No. of slides having CCC ¹	Reliability (%)	CCC per useful slide
Paradichlorobenzene	6	4	67	6.75
Bromonaphthalene	5	4	80	6.25
Combination of both	4	3	75	7.66

¹ Roots were digested 90 minutes at 37°C with a solution of 5.0% pectinase and 2.5% cellulase.

Fixation The optimum time of fixation with acetic acid and ethanol (1:1) was about 1 hour. The tissues gradually hardened with increased duration and at 10 hours, no usable cells were observed. Carnoy's II fixation solution cleared the cytoplasm well and extended the time for fixation.

Discussion

We found that roots from the first seeds to germinate among a group were the most satisfactory for chromosome preparation. Late germinating seeds required varying durations of enzyme treatment and the results were less satisfactory. The optimum length of roots was about 1.5 centimeters. Few actively dividing cells were found in the radical when it first protruded from the seed. Uniformity of length and age of the roots decreased variation of response to the enzymes. Roots developed from regenerated plants remained suitable for treatment over a longer period of time than those developed from seed.

Pretreatment of roots with a saturated solution of paradichlorobenzene and bromonaphthalene for more than 8 hours hardened the roots so, digestion by the enzymes was difficult. When paradichlorobenzene was used alone, pretreatment could be extended to 12 hours with no deleterious effects on enzyme digestion. Carnoy's II fixation solution cleared the cytoplasm well and allowed a longer time for fixation.

Since enzymes easily lose their activity, we stored the enzyme preparation in 2-ml vials at -10°C or lower. Thus, it was unnecessary to melt all of the supply when only a small amount was needed.

We washed the roots 3 to 4 times to provide an appropriate condition for enzyme digestion. It was critical to allow sufficient time for digestion by enzymes, so the cells would be well separated and the chromosomes spread on a single focal plane. The source of enzymes

affected results, so the concentration and ratio of pectinase to cellulase would need to be adjusted when the sources differed from ours.

We removed the desired portion of the root from the enzyme solution by clipping with fine forceps. The root tip was subsequently placed in contact with the surface of a dry slide. If the tip adhered to the slide and separated from the root proper, it usually was adequately digested.

The cells treated with enzymes were very fragile and the preparation could not be tapped to separate the cells after the cover glass was added. After the cover glass was semi-permanently sealed, slight tapping was occasionally helpful.

Abstract

We used root tips of annual *Medicago disciformis* DC. ($2n=2x=16$), perennial *M. sativa* L. 'Lahontan' ($2n=4x=32$), and plants of Lahontan that were regenerated from anther calluses to test various enzyme treatments for root tip chromosome preparation. These *Medicago* entries responded similarly to the types of enzymes and duration of treatment. Treatment with an enzyme solution (5.0% pectinase and 2.5% cellulase) for a duration of 60 to 90 minutes at 37°C gave the best results. The reliability of this treatment and the number of chromosome-countable cells was high. Distribution of cells and spreading of chromosomes in a cell on a focal plane was easily achieved. Treatment periods of less than 1 hour resulted in incomplete digestion. When the digestion time exceeded 90 minutes, many cells were too fragile to be processed. Either pectinase or cellulase alone failed to completely digest the roots, even after treatment for 5 hours. A solution comprised of 5% pectinase and 5% cellulase was so drastic that controlling the treatment time became very critical and difficult.

Key words: Alfalfa, chromosome-preparation, pectinase, cellulase

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