Legume Research, 40(5)2017 : 830-835
Print ISSN:0250-5371 / Online ISSN:0976-0571

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com/www.legumeresearch.in

Caucasian clover (*Trifolium ambiguum* Bieb.) × white clover (*T. repens* L.) – interspecific hybrids developed through tissue culture

Fan Huang[1,2], Mingjiu Wang[2*] and Zhiyong Li[1*]

Grassland Research Institute,
Chinese Academy of Agricultural Sciences, Hohhot-010 010, China.
Received: 13-09-2016 Accepted: 17-02-2017 DOI:10.18805/LR-317

ABSTRACT

In order to create new forage variety, caucasian clover (*Trifolium ambiguum* Bieb.) with strong tolerance to drought and cold introduced from New Zealand as the female parent was hybridized with a white clover (*T. repens* L.) collected from northeast China, which has strong nitrogen fixation capacity. Hybrid embryos of caucasian clover×white clover were successfully raised using embryo rescue. 13.8% of these embryos grew to maturity. The successfully grown plants were transferred to sterilized vermiculite after 5-6 weeks and then to field after 3-4 months. The hybrid plant showed intermediate morphological features and a chromosomal number of 2n=5X=40. In order to obtain more plants for backcross, a tissue culture system was established for the hybrids, using MS+0.1mg l^{-1} 2, 4-D+2mg l^{-1} 6-BA, MS +0.5mg l^{-1} NAA+1mg l^{-1} 6-BA+1mg l^{-1} KT and 1/2MS as the medium for bud-induction, differentiation and root-induction respectively. 80% of the cultured plantlets survived these growing conditions. Hybridity of the plant was also confirmed by ISSR markers using 16 primers.

Key words: Caucasian clover, Embryo rescue, ISSR markers, Tissue culture, White clover, Interspecific hybrid.

INTRODUCTION

Caucasian clover (*Trifolium ambiguum* Bieb.) is a perennial legume forage, with strong drought and cold resistance characteristics, which may in part be conferred by its original habitat and strong rhizomatous growth (Coolbear *et al.*, 1994). However, it is slow to establish (Hill and Mulcahy, 1995; Taylor and Smith, 1998) and requires special rhizobia for nitrogen fixation (Hely, 1963; Zorin *et al.*, 1976) which affects its growth in locations outside its area of origin. White clover (*T. repens* L.) is one of the most important and perennial forage legume in the world. It produces forage of high quality and in root nodules, fixes atmospheric nitrogen, but poor tolerance to drought and cold because of genetic characteristics and its shallow root system (Frame and Newbould, 1986). Although specific Caucasian clover rhizobium for planting inoculation in acidic soil has been successfully isolated and cultured in New Zealand, the validity of this rhizobia varies greatly under different soil conditions, especially in the northern locations where alkaline soils dominate (Patrick and Lowther, 1995; Cherney, 2000). Tetraploid and hexaploid Caucasian clover were introduced from New Zealand into Hohhot, Inner Mongolia, China in 2000. The studies of biology, ecology, physiology and other characteristics showed that the drought and cold tolerance and the growth of hexaploid caucasian clover were significantly better than those of tetraploid Caucasian clover. However, neither hexaploid nor tetraploid strains have nitrogen fixing nodules. Hybridizations between hexaploid caucasian clover and white clover offers a possible source of increased variability that drought-tolerant strains of clover with strong nitrogen fixing performance in western region of Inner Mongolia may be selected.

Plant tissue culture techniques are the important biotechnological tools in forage improvement programs (Mathiyazhagan *et al.*, 2013; Barpete *et al.*, 2014). Tissue culture like embryo rescue have been applied extensively to reproductive battier overcoming of clover and other species (Roy *et al.*, 2004; Barikissou and Baudoin, 2012). The use of interspecific hybridization and tissue culture have been successfully developed to produce F_1 hybrids between tetraploid caucasian clover and white clover in order to combine the superior characteristics of both clovers (Ferguson *et al.*, 1990; Anderson *et al.*, 1991). A range of backcross hybrids, using white clover as the recurrent parent, has been generated and by the third generation of backcrossing, plants that are very similar to white clover but with rhizomes as well as stolon have been produced (Abberton *et al.*, 1998). Little has been reported about hybrids between hexaploid caucasian clover and white clover (Williams, 2014). In this paper, the results of efforts to develop interspecific hybridization between hexaploid Caucasian clover and white clover using tissue culture methods are reported. Backcrossing, which aims to restore fertility to the hybrids, will be performed in subsequent research.

*Corresponding author: zhiyong li 1216@126.com
[1]Grassland Research Institute, Chinese Academy of Agricultural Sciences, Hohhot-010 010, China.
[2]College of Grassland, Resources and Environmental, Inner Mongolia Agricultural University. 010010 Hohhot, China.

MATERIALS AND METHODS

Plant material: White clover (2n=4x=32) seeds were obtained from a wild population collected from the northeast China. The caucasian clover variety monaro (2n=6x=48) was derived from New Zealand. Seeds of both species were sown in April 2010, keeping a row-to- row and plant-to-plant distance of 50 and 50 cm respectively, there were 30 plants as male or female parents of each species.

Pollination and embryo culture: Crossing was done between 7.00 and 9.00 a.m. by hand pollination using caucasian clover as the female plant and white clover as the male plant as significant ovary development occurred only when caucasian clover was used as the female parent (Yamada and Fukuoka, 1985). Flowers were not emasculated before pollination because Caucasian clover is highly self-incompatible (Meredith *et al.*, 1995). Flowers were bagged in parchment bags when at budding stage. After pollination, the flowers were also covered with parchment bags and marked with the date and number. Part of the flowers were left to allow seed to set under natural conditions, while the other part was used for the embryo transfer process.

Pods were removed 12-14 days after pollination and were sterilized before being put into the culture medium. They were rinsed three times using 70% ethanol for 3 minutes each time, and then the surface was sterilized with 10% hypochlorite sodium for 6 minutes, after which they were washed with sterile water. The embryos were then dissected from the ovaries and cultured on a modified MS medium containing 2.5% sucrose and 0.7% agar without any hormones added. The embryos were kept in an automatic light incubator and cultured at 25±2°C under a 12/12-hour (light/dark) photoperiod. Plantlets were transferred to the small plastic pots after 5-6 weeks. After 3-4 months the plantlets were transferred into field. Morphology of the plants was observed by qualitative assessments.

Observation of root nodules and nitrogen fixation: Hybrids that showed vigorous growth were selected for observation of root nodules. Acetylene reduction assay (ARA) was used for measurement of nitrogen fixation.

Chromosome counts: Mitotic chromosome counts were made on root tips pre-treated for 24 hours at 0° C in ice cold distilled water, then fixed for 12 hours in 3:1 ethanol-acetic acid, cell dissociation for 8-10 minutes in 1N hydrochloric acid at 60!, stained with carbolic acid and magenta.

Pollen fertility: Pollen fertility was assessed as the percentage of full stainable grains with normal morphology by the double staining method of Owczarzak (1952). A total of 100 grains were scored per plant from at least 3 flowers.

Establishment of regeneration system: Stems of the F_1 hybrids were used as the explants to culture adventitious buds. They were cut into small pieces about 1cm long and inoculated on a bud-induction medium consist of MS medium supplemented with 2, 4-D 0.1mg l^{-1}, 6-BA 2mg $l^{-1,}$ 2% sucrose and 0.7% agar. The pH was adjusted to 6.0. After two subcultures the calluses were transferred onto a differentiation medium, which was MS medium supplemented with NAA 0.5mg l^{-1}, 6-BA 1mg l^{-1}, KT 1mg l^{-1}, 2% sucrose and 0.7% agar, pH 6.0. When shoots were longer than 5cm, the plants were inserted into rooting and plantlets hardening medium, which was ½ MS medium without any supplements.

Molecular analysis: Total genomic DNA was extracted from the leaves of individual plant using a plant genome kit. 16 ISSR primers were screened for their ability to detect the genetic relationships among the 3 plant materials and tissue

Table 1: ISSR primers used, annealing temperature (Ta), total bands (TB), polymorphic bands (PB) for each primer, and the percentage of polymorphic bands (PPB)

Primer	Sequence(5'-3')	Ta (^{O}C)	TB	PB	PPB (%)
S2	(AG)8GC	56	13	10	76.92
S7	(Ac)8CG	56	11	8	72.72
S10	(GA)8CC	56	12	9	75.00
S20	(TC)8CC	52	6	4	66.67
S22	(CT)8G	52	5	4	80.00
S25	(CCG)6	72	8	7	87.50
S27	(AG)8AC	54	14	12	85.71
S28	(AG)8TC	54	11	10	90.91
S30	(AG)8GA	54	7	7	100.00
S33	(AC)8GT	54	10	8	80.00
S34	(AC)8CT	54	9	6	66.67
S35	(AC)8AG	54	12	10	83.33
S37	(GA)8CT	54	10	8	80.00
S40	(GA)8CA	54	13	10	76.92
S41	(CT)8A	50	9	6	66.67
S43	(C T)8TC	54	6	4	66.67
Total			156	123	78.85

Table 2: Genetic similarity and genetic distance of tested plants

Materials	T. ambiguum Bieb.	T. repens	Hybrid plants no.25	Regeneration plantlet
T. ambiguum Bieb.	—	0.3563	0.5843	0.5798
T. repens	0.6437	—	0.3115	0.3057
Hybrid plant no.25	0.4157	0.6885	—	0.9565
Regenerative plantlet	0.4202	0.6943	0.0435	—

Fig-1: Two plant types of F1 plants: prostrate(upper figure) and erect(lower figure) (×0.2)

culture regeneration F1 plants (Table 1). Amplifications were performed with the following procedures: preliminary denaturation at 94°C for 2 min; 30 cycles each involved denaturation at 94°C for 30s, anneal at Ta (Table 1) for 45s, extended at 72°C for 1 min, final incubation for 7 min at 72°C was performed to ensure that the primer extension reaction preceded to completion. Amplified products were separated by electrophoresis on a 1.5 % (w/v) agarose gel using 1×TBE buffer. Photo documentation was performed under UV light using a photo imaging system.

Data scoring and statistical analysis: The number of bands generated by different primers was scored and the frequency of the polymorphisms was calculated. The presence or absence of bands was scored in a binary matrix that used '1' for presence and '0' for absence. A similarity matrix for each sample was determined. The Pop gene software (version 1.31) was used to calculate genetic distance.

RESULTS AND DISCUSSION

In total, 4501 florets pollinated. Dissection of 1253 ovaries revealed only 384 embryos that were healthy with swelling. Of these, 53 matured in culture, indicating that the frequency of embryo production was only 8.5% of the crossed flowers and that 13.8% of all embryos were survived.

Hybrid embryos were observed 6-14 days after pollination. There was no germination of the hybrid embryos in culture between 6 and 11 days, and all germination of embryos in culture occurred between 12 and 14 days. Hence, dissection of embryos was carried out between 12–14 days after pollination.

A total of 384 embryos were cultured of which 91 (23.70%) showed growth within 15–20 days after inoculation. 38 of these eventually died during subculture. Williams (1978) noted that only 30 embryos from many hundred pollinated flowers were large enough for dissection and only 6 showed any growth in culture. In other research on hybridization between *Trifolium alexandrinum* and *T. resupinatum*, out of 556 crossing attempts, a total of 26 ovules were recovered (Malaviya *et al.*, 2004a; Kaushal *et al.*, 2005). In our study, after nearly 4-5 months of culturing *in vitro*, the remaining 53 were transferred to vermiculite.

Thirteen of 53 plants were different from their parents in morphology, and the remaining 40 showed vigorous growth but more like Caucasian clover and did not show any significant difference with parents. More detail will be described when the hybridity of the 40 plants have been confirmed. The 13 F1 plants showed two kinds of plant type: prostrate and erect (Fig-1). There were 8 prostrate plants and 5 erect plants, but only one has flowered (plant No. 25). The hybrid plant No.25 was vegetatively intermediate to both parents. In research on *T. alpestre* × *T. pretense*, Phillips et al. (1992) observed that the recovered hybrid was morphologically intermediate between the two parents. Yamada et al. (1989) reported that hybrid plants showed traits intermediate between the two parents with poor field performance and very low fertility. In our study, hybrid plant No.25 was erected in nature and with rhizomes, but no stolon was observed (Fig-2).

Fig-2: Caucasian clover ×white clover hybrid plant (center) compared with the Caucasian clover female parent (right) and white clover male parent (left) after two years of growth.(×0.06)

Fig-3: Leaves of hybrid plant no.25 (center) compared with leaves of the Caucasian clover female parent (right) and white clover male parent (left).(×0.3)

Fig-4: Flowers of hybrid plant no.25 (center) compared with flowers of the Caucasian clover female parent (right) and white clover male parent (left).(×1.0)

Fig-5: Root nodules of hybrids compared with root nodules of the Caucasian clover female parent (right) and white clover male parent (left).(×0.3)

The leaflets of hybrid plant No.25 were broadly ovate and were distinctly wider in proportion to length unlike the ovate leaflets of the female parent and the obovate leaflets of the male parent. Abberton *et al.* (1998) also found that the ratio of leaflet length to width in Caucasian clover was greater than in white clover, with the F1 hybrid's ratio intermediate between the two levels. Leaves of the hybrids were a lighter green color than those of both parents. Leaves of hybrid NO.25 showed the dominant white leaf- V mark, which was intermediate in shape between those of the two parents (Fig-3). The flowers of the hybrid NO.25 was similar to those of white clover in size. Inflorescence of the hybrid was a raceme shape, which is same as Caucasian clover (Fig-4).

Root nodules were found on the hybrid NO.25 (Fig-5). They were irregular in shape, and showed white or pale yellow color, and only a relatively small number existed in shallow soil. The result of the ARA indicated that the nitrogen fixating nodules of hybrid NO.25 was still absent. Widdup *et al.* (2003) reported that all the hybrid plants were nodulated irrespective of strain of rhizobia used for inoculation.

100 somatic cells from the root tips of hybrid NO.25 and both parents were observed for chromosomal associations during mitosis. The female parent Caucasian clover revealed 2n=6X=48 and the male parent white clover had 2n=4X=32 chromosomes. Hybrid NO.25 showed 2n=5X=40 chromosomes, providing good evidence for hybridity (Fig-6).

Pollen grains of hybrid No.25 were hollow and not stained, while those of the female parent and male parent were red and round (Fig-7). The hybrid No.25 is highly sterile with pollen fertility of 0.63% in the natural flowering season. No seeds were obtained under natural conditions.

The purpose of tissue culture is to obtain more plants for backcrossing during the next flowering season. Both ends of the hybrid stems enlarged in about 1 week and produced callus after 20 days. The colors of calluses were light green or green. Vigorous calluses were transferred to a differentiation medium and after two subcultures. Several adventitious buds were formed after 30 days. Most of the plantlets did not develop roots in the differentiation medium. Roots developed in 1/2MS medium were robust and easily established. For the purpose of hardening plantlets, the 1/2MS medium was also used for subculture after rooting. 85% of the plantlets survived acclimatization to surrounding conditions and established successfully in the greenhouse.

16 ISSR primers were selected as suitable for PCR (Table.1). On average, 9.75 bands were recorded per primer. 123 (81.18 %.) of the 156 bands were polymorphic among the 4 samples. Parents and hybrid No.25 could be distinguished from each other clearly by each primer (Fig.8).

The genetic distance was relatively large between caucasian clover and white clover, but small between the hybrid NO.25 and its regeneration plantlets. This illustrates significant genetic differences between both parents, and little variance in the regeneration system of hybrids. Genetic distance between hybrid NO.25 and female parent was 0.4157, and 0.6885 between hybrid NO.25 and male parent (Table.2), indicating that hybrid NO.25 tend towards the female parent more than the male parent.

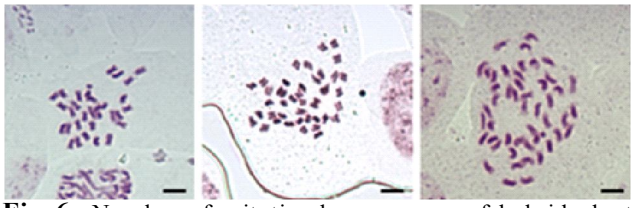

Fig-6: Number of mitotic chromosomes of hybrid plant 2n=5X=40 (center) compared with the Caucasian clover female parent 2n=6X=48 (right) and white clover male parent 2n=4X=32 (left). Bar: 5µm

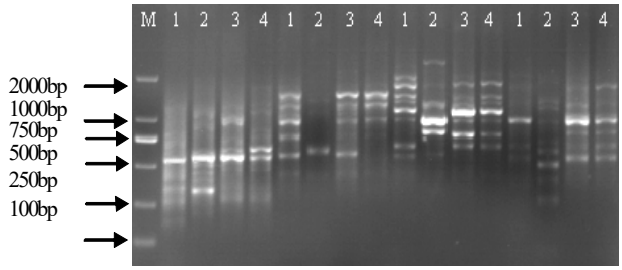

Fig- 8: ISSR result of 4 tested plants with primers S10, S20, S33 and S41. 1 Caucasian clover female parent, 2 white clover male parent, 3 hybrid plant, 4 regenerative plantlet. M: markers (from top to bottom) are 2000, 1000, 750, 500, 250, and 100 bp.

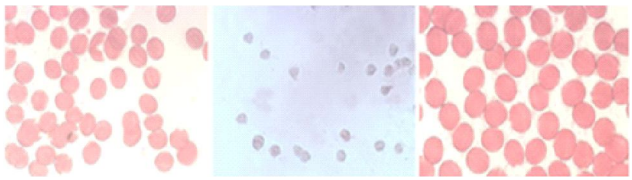

Fig-7: Pollen fertility of hybrid plant no.25 (center) compared with pollen fertility of the Caucasian clover female parent (right) and white clover male parent (left)

In Inner Mongolia, most clover that can be utilized directly for grazing cannot survive due to cold, drought and other harsh natural conditions. Introduction and breeding of clover varieties that can adapt to condition in Inner Mongolia has become an important issue. caucasian clover is the only long-lived clover with long underground rhizomes and strong reproductive capacity. There are good prospects of caucasian clover whether used as a cultivar or for grazing. Much hope is placed on efforts to produce hybrids between caucasian clover and white clover with stress tolerance and nitrogen fixing, and provide suitable variety for clover cultivation in Inner Mongolia.

To date, only an agronomically weak form of 4x Caucasian clover of Turkish origin has been crossed with white clover by application of embryo and ovule culture to form fertile progeny only in very low numbers: one by Williams and Verry (1981), two by Meredith *et al.* (1995) and two by Williams et al. (2013). There have been other reports where only sterile hybrids were obtained (Williams, 1978; Yamada *et al.*, 1989). It has been possible to create hybrids indirectly by crossing 6x Caucasian clover with 6x hybrids to produce complex 6x hybrids (Williams *et al.*, 2006b). These were strongly rhizomatous but very low in fertility (Williams *et al.*, 2013) and they have not been further developed (Williams, 2014).

In this research, the authenticity of the hybrid existence by direct crossing was confirmed with morphological, cytological characterization and DNA molecular analysis. To our knowledge, this study has resulted in development of the first interspecific hybrid between hexaploid Caucasian clover and white clover. It was also the first work combining morphological, cytological and ISSR analysis to determine the differences between hybrid and parents.

ACKNOWLEDGEMENT

We thank Prof. Fengling Shi for advice, and we are also grateful for the assistance of the pratacultural science state key discipline green house. This work was supported by the National Nature Science Foundation of China (No.31260577).

REFERENCES

Abberton, M.T., Michaelson-Yeates, T.P.T, Marshall, A.H, Holdbrook-Smith, K. and Rhodes, I. (1998). Morphological characteristics of hybrids between white clover, *Trifolium repens* L., and Caucasian clover, *Trifolium ambiguum* M. Bieb. *Plant Breeding* **117**: 494-496.

Anderson, J.A., Taylor, N.L. and Williams, E.G. (1991). Cytology and fertility of the interspecific hybrid *Trifolium ambiguum* × *T. repens* L. and backcross populations. *Crop Sci.* **31**: 683-687.

Barikissou, E. and Baudoin, J. (2012). A review of embryos rescue in food legumes in general and in the genus Phaseolus in particular. *Legume Research* **16**(2):203-215.

Barpete, S., Sharma, N.C. and Kumar, S. (2014). Assessment of somaclonal variation and stability in in vitro regenerated grasspea plants using. *Legume Research* **37**(4):345-352.

Cherney, J.H. 2000. Kura clover for New York pastures. Kura Clover Update, August, (2000). Cornell University

Coolbear, P., Hill, M.J. and Efendi, F. (1994). Relationships between vegetative and reproductive growth in a four year old stand of Caucasian clover (*Trifolium. ambiguum* M Bieb.) cv. Monaro. *Proc. Agron. Soc. N. Z.* **24**, 77–82.

Ferguson, N.H., Rupert, E.A. and Evans, P.T. (1990). Interspecific *Trifolium* hybrids produced by embryo and ovule culture. *Crop Sci.* **30**: 1145-1149.

Frame, J. and Newbould, P. (1986). Agronomy of white clover. *Adv. Agron.* **40**: 1-88.

Hely, F.W. (1963). Relationship between effective nodulation and time to initial nodulation in a diploid line of *Trifolium ambiguum* M. Bieb. *Australian Journal of Biological Sciences* **10**: 1-16.

Hill, M.J. and Mulcahy, C. (1995). Seedling vigour and rhizome development in *Trifolium ambiguum* M. Bieb. (Caucasian clover) as affected by density of companion grasses, fertility, drought, and defoliation in the first year. *Aust. J. Agric. Res.* **46:** 807-819.

Kaushal, P., Malaviya, D.R., Roy, A.K., Kumar, B. and Tiwari, A. (2005). *Trifolium alexandrinum* × *T. resupinatum* –interspecific hybrids developed through embryo rescue. *Plant Cell, Tissue and Organ Culture* **83**: 137-144.

Malaviya, D.R., Roy, A.K., Kaushal, P., Kumar, B. and Tiwari, A. (2004a). Development and characterization of interspecific hybrids of *Trifolium alexandrinum* × *T. apertum* using embryo rescue. *Plant Breeding* **123:** 536-542.

Mathiyazhagan, S., Pahuja, S.K. and Ahlawat, A. (2013). Regeneration in cultivated (*Cyamopsistetragonoloba* L.) and wild species (*C. serrata*) of guar. *Legume research* **36**(2):180-187.

Meredith, M.R., Michaelson-Yeates, T.P.T., Ougham, H.J. and Thomas, H. (1995). *Trifolium ambiguum* as a source of variation in the breeding of white clover. *Euphytica* **82**: 185-191

Owczarzak, Alfred. (1952). A rapid method for mounting pollen grains with special regard to sterility studies. *Stain Technology* **27**: 249-251.

Patrick, H.N. and W.L Lowther. (1995). Inoculation of the rhizobia on the nodulation and establishment of *Trifolium ambiguum*. *Soil Biol. Biochem.* **27**(4/5): 717-720.

Phillips, G.C., Collins, G.B. and Taylor, N.L. (1982). Interspecific hybridization of red clover (*Trifolium pretense* L.) with *T. sarosiense* Hazsl. using in vitro embryo rescue. *Theor. Appl. Genet.* **62**: 17-24.

Phillips, G.C., Grosser, J.W., Berger, S., Taylor, N.L. and Collins, G.B. (1992). Interspecific hybridization between red clover and *Trifolium alpestre* using invitro embryo rescue. *Crop Sci*. **32**: 1113-1115.

Roy, A.K., Malaviya, D.R., Kaushal, P., Kumar, B. and Tiwari, A. (2004). Interspecific hybridization of *Trifolium alexandrinum* with *T. constantinopolitanum* using embryo rescue. *Plant Cell Rep.* **22**: 705-710.

Taylor, N.L. and Smith, R.R. 1998. Kura clover (*Trifolium ambiguum* M. B.) breeding, culture, and utilisation. *Adv. Agron.* **63**: 154-179.

Widdup, K.H., Hussain, S.W., Williams, W.M., Lowther, W.L., Pryor H.N. and Sutherland B.L. (2003|). The development and plant characteristics of interspecific hybrids between white and Caucasian clover. 'In Legumes for Dryland Pastures. Proceeding New Zealand Grassland Association Symposium'. *Grassland Research and Practice Series NO.***11**: 143-148.

Williams, E. (1978). A hybrid between *Trifolium repens* and *T. ambiguum* obtained with the aid of embryo culture. *New Zealand J. Bot.* **16**: 499-506.

Williams, E. and Verry, I.M. (1981).A partially fertile hybrid between *Trifolium repens* and *T. ambiguum*. *New Zealand J. Bot.* **19**: 1-7.

Williams, W.M., Verry, I.M., Ansari, H.A. and Hussain, S.W. (2006 b.) First backcrosses of caucasian x white clover hybrids to caucasian clover. In 'Breeding for success: diversity in action. Proceedings of the 13th Australasian Plant Breeding Conference'. NewZealand977–983.

Williams, W.M., Verry, I.M., Hussain, S.W., Ansari, H.A., Widdup, K.H., Ellison, N.W. and Nichols, S.N. (2013). Widening the adaptation of white clover by incorporation of valuable new traits from wild clover species. In 'Proceedings of the 22nd International Grasslands Congress'. Sydney. 287–289.

Williams, W.M. (2014). *Trifolium* interspecific hybridization: widening the white clover gene pool. *Crop & pasture science* **65:** 1091-1106.

Yamada, T. and Fukuoka, H.(1985). Application of ovule culture to interspecific hybridisation between *Trifolium repens* and *T. ambiguum*. In Proceedings of the XV Int Grassl Conf:241-243.

Yamada, Y., Fukuoka, H. and Higuchi, S. (1989). Interspecific hybridisation of tetraploid Kura clover (*Trifolium ambiguum* M. Bieb.) and white clover (*T. repens* L.) using ovule culture. *J. Jap. Soc. Grassl. Sci.* **35**: 180-185.

Zorin, M., Brockwell, J. and Muller, W.J. 1976. Symbiotic characteristics of *Trifolium ambiguum* seedlings grown in tube culture as affecting subsequent symbiotic vigour. *Australian Journal of Experimental Agriculture and Animal Husbandry* **16:** 854-862.