From Forest Nursery Notes, Summer 2011

140. Topographic tetrazolium testing of black walnut (*Juglans nigra* L.) seeds.
Flores, P. C., Poggi, D., Garcia, S. M., and Gariclio, N. F. Seed Science and Technology 39:230-235. 2011.

Flores, P.C., Poggi, D., García, S.M. and Gariglio, N.F. (2011), *Seed Sci. & Technol.*, **39**, 230-235

Research Note

Topographic tetrazolium testing of Black Walnut (*Juglans nigra* L.) seeds

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(Accepted November 2010)

Summary

The aims of this work were (1) to propose a topographic tetrazolium (TZ) test methodology for *Juglans nigra* seeds by adjusting the wide range of incubation times recommended for *Juglans sp.*, (2) to describe the staining patterns of viable and non-viable seeds, and (3) to establish the relationship between the results of the TZ test and the germination test. Decreasing incubation time from 48 to 4 hours resulted in the best coloration of the seeds. Four staining patterns were observed in viable seeds whereas one pattern accounted for over 95% of the non-viable seeds. Viable seeds which were cold-stratified had a germination of 96%, resulting in a high correlation between the results of the TZ viability test and those from the germination test ($r = 0.98$) and other indices indicating the rate of germination, namely the germination velocity index ($r = 0.89$) and the medium time of maximum germination ($r = -0.95$).

Experimental and discussion

In Argentina, black walnut (*J. nigra*) is the main rootstock used for vigorous varieties of common walnut such as the cv. Franquette. *J. nigra* rootstock plus cv Franquette provides an excellent pollinator for California varieties such as Chandler, Hartley and Tulare (Iannamico, 2009). Presently, *Juglans nigra* L. is propagated by seed; however, 3 to 4 months are required to determine the proportion of germinable seeds due to the need to breaking physiological dormancy (Baskin and Baskin, 2004) by means of cold stratification (FAO, 1991; Ellis *et al.*, 1985). The tetrazolium (TZ) test is a rapid viability test which allows interpretation of seed viability according to the staining pattern of seed tissues (Bonner, 1974; Moore, 1985; ISTA, 1991). Every species requires its own specific instructions for handling, preparation, imbibition, dissection and evaluation of the TZ test (Enescu, 1991; Gosling, 2004).

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In the case of black walnut there are general recommendations for the genus *Juglans*, which shows a wide range of incubation time (16–48 hours) (AOSA, 2000); however, this protocol has not given adequate results in our previous experience.

The sensitivity of the TZ test in predicting viability would be confirmed if its results were correlated with a germination test or with other indices such as the germination velocity index (GVI) and the medium time to maximum germination (MTMG).

The aims of this work were (1) to propose a topographic TZ test methodology for *Juglans nigra* seeds by adjusting the wide range of incubation times recommended for *Juglans sp.*, (2) to describe the staining patterns of viable and non-viable seeds, and (3) to establish the correlation between the results of the TZ test and the germination test. A random sample of mature seeds were obtained from 30 trees of *J. nigra* grown at the J.F. Villarino Experimental Field of the Facultad de Ciencias Agrarias of the Universidad Nacional de Rosario, located in Zavalla (33°01'S,60°53'W), province of Santa Fe, Argentina. After removal of the meso and exocarp, nuts were disinfected for 2 min with sodium hypochlorite solutions (2% v/v) (Alzugaray *et al.*, 2006) and rinsed with distilled water. Seeds were then divided into two groups; one half of the seeds were soaked in water for 24 h; afterwards, they were stratified in a chamber at 5°C for 4 months. During stratification samples of 50 seeds were wrapped with moist paper and placed into a black plastic bag to maintain moisture. The other half of the seeds was stored at room temperature (20°C). Nuts were completely immersed in water overnight at room temperature prior to performing the TZ test.

The aim of the first experiment was to adjust the time of incubation to TZ. Four timings of exposure were used 48 h, 24 h, 10 h, and 4 h. A solution of TZ (2,3,5 triphenyl tetrazolium chloride) at 1% was used at 33°C in darkness. Nuts were cut longitudinally using a scalpel, without separating the seeds from the woody endocarp, then immersed in the TZ solutions. Nuts were washed with distilled water after incubation (ISTA, 1991). For the initial evaluation of seeds, the reference model proposed by AOSA (2000) for *Juglandaceae* family was used. Viable seeds are those that show completely stained radicle, hypocotyl, epicotyl, and cotyledons. Also considered viable are those seeds that show no more than one-third of the distal end of the cotyledons unstained. Nuts are considered as non-viable or abnormal when they do not show any staining, or when less than two-thirds of the distal end of the cotyledons remains unstained (AOSA, 2000). The staining images were digitally captured using an HP Scanjet 2400 scanner with a resolution of 300 DPI.

In the second experiment the staining patterns of viable and non-viable nuts were described. Seeds were conditioned as in the previous experiment but the time of incubation to TZ used was that which gave the best results in the previous experiment. Both four-month stratified and non-stratified seeds were used. Each staining pattern was characterized and quantified, establishing its relative proportion.

A complete randomly experimental design with four replications of 50 seeds per treatments was used in the first and the second experiments. The two halves of the cut seed were used in both experiments.

In the third experiment, control seeds (untreated with TZ) and seeds following staining in the TZ test in the second experiment were used to establish the correlation between

the results of the TZ test and the germination test. The germination test was conducted following the specifications of the International Seed Testing Association (2003). In the case of seeds used in the TZ test, only the half section of the cut seeds which had the embryo was assessed. Viable and non-viable seeds were grouped for each staining pattern; afterwards, seeds were placed into plastic trays on sand (40 mm layer) and covered with 20 mm of uncompressed wet sand. Each tray was then enclosed in a transparent plastic bag and placed in the germination chamber (Forma Scientific, Models 3744) for 28 days, with an alternating regime temperature of 20/30°C (16h/8h) in light (Brinkman, 1974). The same procedure was used with control seeds (untreated with TZ) but in this case the whole seed was used.

The GVI was calculated according to Kotowiski (cited by Silva and Nakagawa, 1995), whereas the MTMG was calculated according to Edmond and Drapala (1958).

A complete randomly experimental design was used in the third experiment. Four replications with a variable number of seeds (according to the proportion of each staining pattern shown in the second experiment) were used in the germination test performed with seeds after soaking in TZ solution, whereas four replications of 50 seeds per treatments were used when the germination test was performed to untreated seeds. Data from the TZ test were compared with the result of the germination test and other indices of velocity of germination using linear regressions. Analysis of variance was performed on the data, and means were compared by Tukey's test.

Black walnut seeds incubated in TZ solution for 48 (figure 1a) or 24 (figure 1b) hours showed a dark stain and noticeable tissue deterioration that did not allow a good assessment of the seeds. Shortening the time of incubation to 10 hours (figure 1c) improved the staining but it remained being gloomy and opaque with some areas of the seeds showing tissue deterioration. Further reduction in the incubation time to 4 hours resulted in the best coloration of the seeds (figure 2a–d).

The seminal teguments (testa and tegmen) are physical barriers to the aqueous solution of TZ. However, the procedure to perform the TZ test causes different damage on the seminal teguments according to the protocol for each species. In common walnuts, the hardy endocarp was removed and the seminal teguments were not damaged (ISTA, 2003), whereas in black walnuts the hardy endocarp and the whole nut were longitudinally cut causing the division of the seeds into two halves. Thus, the seminal teguments were also cut allowing a direct exposition of the internal seed tissues to the TZ solution. The absence of barriers to TZ penetration and the mechanical damage of tissues caused by the scalpel may explain the need to reduce the time of incubation of black walnut seed in the TZ test to four hours, instead of the generally recommended 16–48 hours for the genus *Juglans* (AOSA, 2000). The dilution of the TZ concentration or reducing the incubation temperature could also be evaluated, as alternatives to reducing the incubation time in order to improve the staining in the TZ test of black walnuts (Enescu, 1991).

Black walnut seeds showed an average of 77 ± 1.12% of viable seeds in the TZ test. Four staining patterns were observed in viable seeds. The first appeared at a relative frequency of 31.16 ± 3.2%; radicle, hypocotyls, cotyledons and epicotyl were turgid and completely stained with intense and bright red coloration (figure 2a). A similar stain but with faint-pink coloration (figure 2b) was the most common pattern (47.4 ± 4.1%). The

Figure 1. Effect of the time of incubation in tetrazolium salt (1%) on the quality of the coloration obtained in seeds of *Juglans nigra*. Seeds were incubated during 48 h (a), 24 h (b), or 10 h (c) at 33°C.

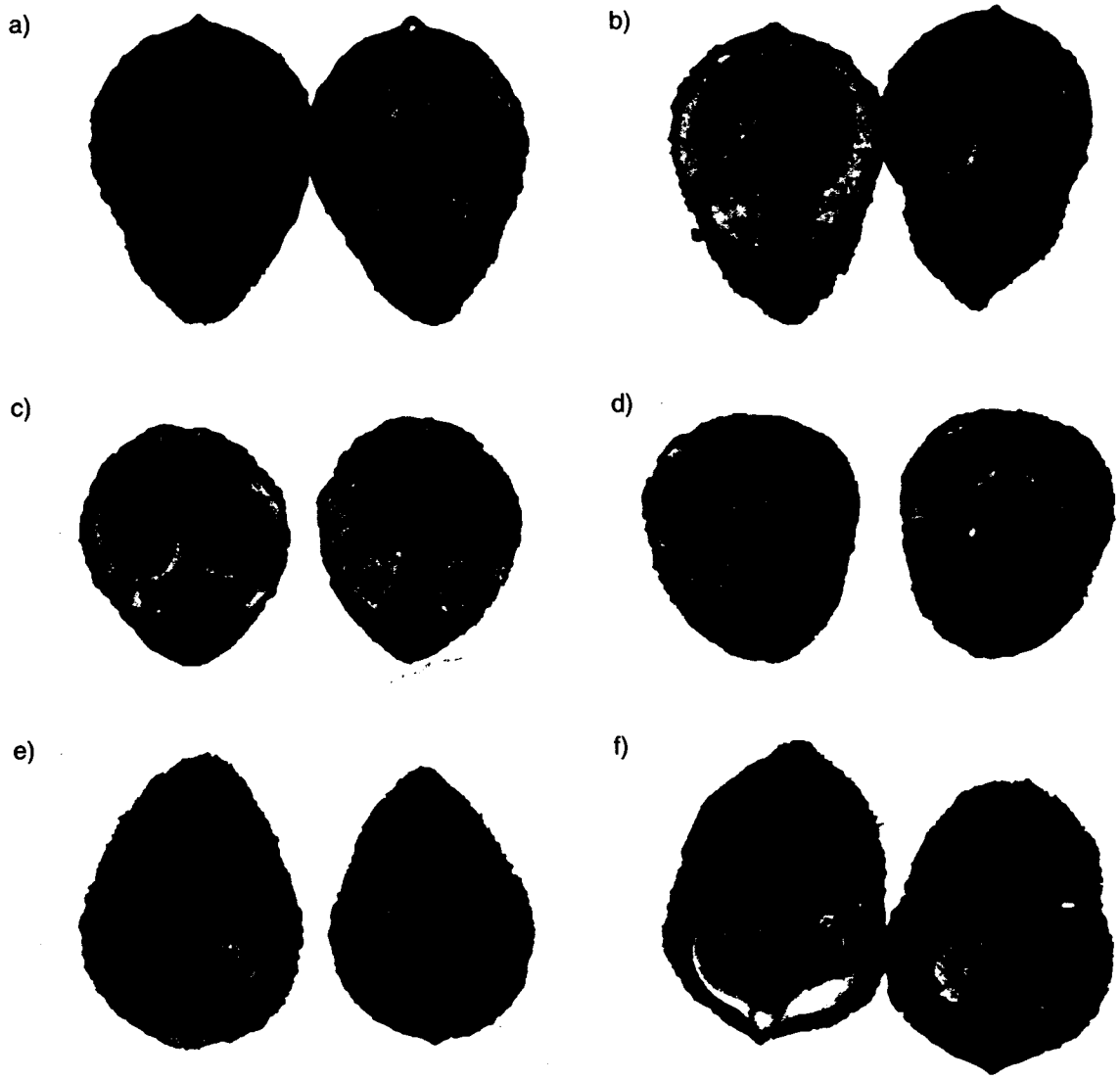

Figure 2. Stain patterns of viable (a-d) and non-viable (e-f) seeds for *Juglans nigra* incubated in the tetrazolium salt (1%) during four hours at 33°C.

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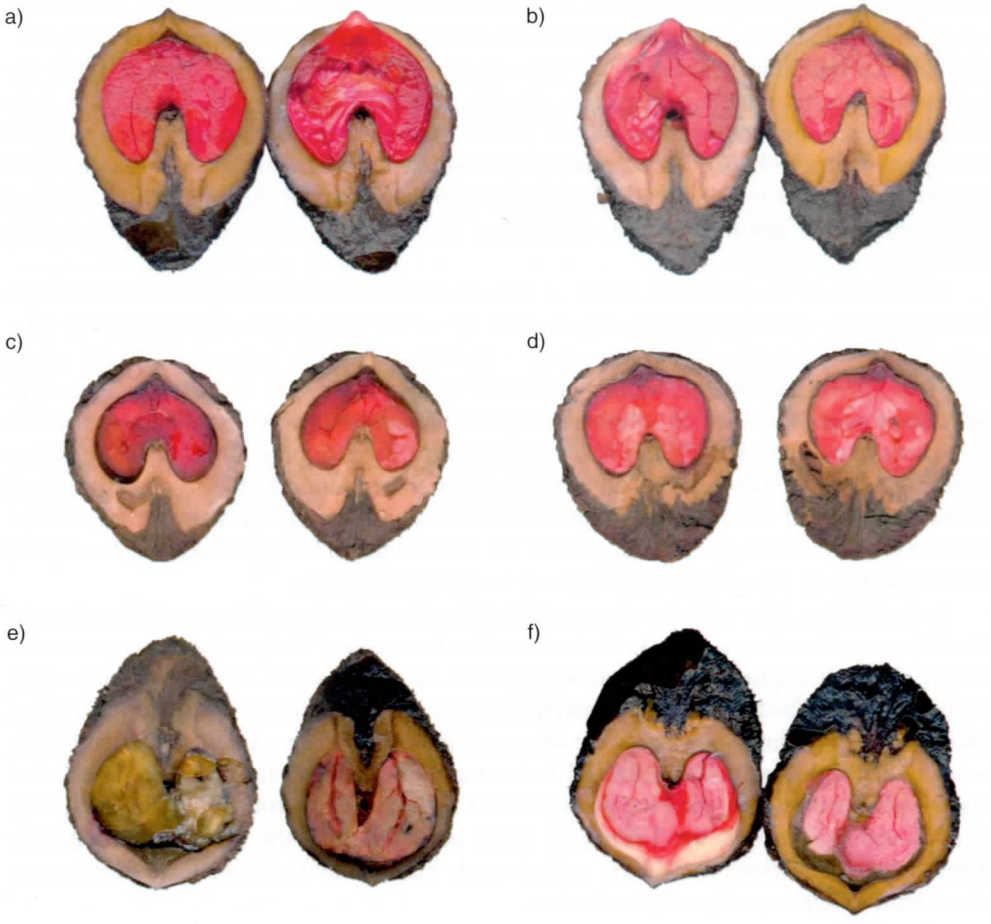

Figure 2. Stain patterns of viable (a-d) and non-viable (e-f) seeds for *Juglans nigra* incubated in the tetrazolium salt (1%) during four hours at 33°C.

third staining pattern showed the radicle completely stained and the cotyledons partially stained at their central and distal side (figure 2c); this was the least common staining pattern (1.3 ± 0.2%). The last pattern was characterized by a non-uniform whole tissue coloration (figure 2d) which made up 20.12 ± 1.9% of the staining patterns of viable seeds.

Non-viable seeds showed non-turgid and deteriorated tissues, with faint yellow-pink colour (figure 2e) probably explained by bacterial infections on the dead tissues (Enescu, 1991). This pattern occurred in over 95% of non-viable seeds. In some cases, the radicle, hypocotyl, epicotyl and at least two-thirds of the cotyledons area were not stained (figure 2f).

Cold-stratified seeds which were shown to be non-viable in the TZ test did not germinate in the germination test. Viable seeds which were not cold-stratified after harvest were also unable to germinate due to the depth of physiological dormancy that characterizes *Juglans sp.* (Nikolaeva, 1977; Baskin and Baskin, 2004). On the other hand, seeds stratified after harvest which were shown to be viable in the TZ test showed a 96% germination rate after soaking in the TZ solutions. The germination rate was 100% for seeds that showed the staining pattern described in the figure 2a, 2b, and 2c, and only seeds that corresponded with the stained pattern shown in figure 2d were unable to reach full germination (80.6%). These results demonstrate the existence of a significant and positive correlation ($r = 0.98$; $P = 0.020$) between the results of the viability TZ test and the results of the germination test, which was also observed previously in other species (Alzugaray *et al.*, 2005).

The results of the germination test of control seeds did not show significant difference with that of the seeds previously soaked in TZ ($P = 0.818$). However, the other GVIs were significantly affected by TZ treatment ($P = 0.0001$). GVI was reduced by 44.2% (from 19.64 to 10.96) and MTMG values increased 94.7% (from 4.98 to 9.70 days) in seeds previously soaked in the TZ solution. These differences might be explained by the fact that seeds exposed to the TZ test had lower reserves because only a half of the seed was used in the germination test and an unknown portion of the remaining reserves was used in the reduction of the TZ compounds. Therefore, a high correlation between the TZ test and the GVI ($r = 0.89$; $P = 0.048$), and the TZ test and the MTMG ($r = -0.949$; $P = 0.025$) were observed in the control seeds but not in TZ-treated seeds.

In conclusion, this work has demonstrated that the reduction of the incubation time in the TZ test to four hours gave the best coloration for seeds of *J. nigra* in comparison with longer times of exposure. Four staining patterns of viable seeds and two of non-viable seeds were described and a high correlation coefficient between the results of the TZ test and that of the germination test were observed ($r = 0.98$). The particular feature of this work is that the seeds used in the TZ test were able to germinate when they were used for the germination test, showing no significant difference in germination compared with control seeds. However, the TZ treated seeds needed double the time for germination. Thus, it was confirmed that black walnut seeds considered viable in the TZ test were those with a high capacity of germination.

References

Alzugaray, C., Carnevale, N., Salinas, A. and Pioli, R. (2005). Observations on quality of *Schinopsis balansae* Engl. seeds. *Seed Technology*, **27**, 49-58.

Alzugaray, C., Carnevale, N., Salinas, A. and Pioli, R. (2006). Calidad de semillas de *Aspidosperma quebracho-blanco* Schlecht. *Quebracho,* **13**, 26-35.

AOSA. Association Official Seed Analysts (2000). *Tetrazolium Testing Handbook.* Contribution N° 29. Las Cruces, Association of Official Seed Analysts.

Baskin, J.M. and Baskin, C.C. (2004). A classification system for seed dormancy. *Seed Science Research,* **14**, 1-16.

Bonner, F.T. (1974). Tests for vigor in cherrybark oak acorns. Proceedings of the Association of Official Seed Analysts, **64**, 109-114.

Brinkman, K.A. (1974). *Juglans* L., walnut, pp. 454-459 *in* Schopmeyer, C.S. (Ed.) *Seeds of woody plants in the United States.* Washington DC, USDA Forest Service, Agricultural Handbook 450.

Edmond, J.B. and Drapala, W.J. (1958). The effect of temperature, sand and soil, and acetone on germination of okra seed. Proceedings of the American Society for Horticultural Science, **71**, 728-734.

Ellis, R.H, Hong, T.D. and Roberts, E.H. (1985). *Handbook of Seed Technology for Genebanks.* Volume II. Juglandaceae. International Board for Plant Genetic Resources, Rome, Italy.

Enescu, V. (1991). The tetrazolium test of viability. 9, 1-7 *in* Gordon, A.G., Gosling, P.G. and Wang, B.S.P. (Eds) *Tree and shrub seed handbook.* ISTA, Zurich, Switzerland.

FAO. (1991). Seed pretreatment. 8, 241-278 In Willan, R.L. (Ed.) *A guide to forest seed handling.* FAO Forestry paper 20/2, FAO, Rome.

Gosling, P.G. (2004). Viability testing. In *Seed Conservation: Turning science into practice.* (eds R. Smith, J. Dickie, S. Linington, H. Pritchard. and R.J. Probert), 24, 445 - 481 (Eds.), Royal Botanic Gardens, Kew, UK

Iannamico, I. (2009). *El cultivo del nogal en climas templados. I. Material vegetal.* Estación Experimental Agropecuaria Alto Valle, Ediciones del Instituto Nacional de Tecnología Agropecuaria.

ISTA. International Seed Testing Association (1991). *Tree and shrub seed handbook.* Gordon, A.G., Gosling, P. and Wang, B.S.P. (Eds.). Zurich, Switzerland.

ISTA. International Seed Testing Association (2003). *International rules for seed testing.* International Seed Testing Association, Zurich, 333pp.

Moore, R.P. (1985). *Handbook on tetrazolium testing.* 1st Edition. International Seed Testing Association. Zurich, Switzerland.

Nikolaeva, M.G. (1977). Factors controlling the seed dormancy pattern, pp. 51-74 *in* Khan, A.A. (Ed.) *The physiology and biochemistry of seed dormancy and germination.* New York, Elsevier.

Silva, J.B. and Nakagawa, J. (1995). Estudo de fórmulas para cálculo da velocidade de germinaçao. *Informativo ABRATES,* **5**, 62-73.