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# Effect of sulfuric acid pretreatment on breaking hard seed dormancy in diverse accessions of five wild *Vigna* species

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## Summary

Many *Vigna* species are important in agriculture, but a high content of hard seeds usually present which create the difficulties in seed testing and sowing. The effectiveness of the pretreatment periods (0, 3, 6, 9, 12 and 15 min.) for breaking dormancy of hard seeds using concentrated sulfuric acid was investigated for 18 seed lots of 14 accessions of 5 wild *Vigna* species (V. *membranacea, V. oblongifolia, V. racemosa, V. schimperi* and V. *vexillata).* Most pretreatments significantly ($P < 0.05$) reduced hard seed content and improved germination percentage and germination rate when compared to untreated seeds. Effectiveness of the pretreatment improved as the pretreatment period increased from 3 to 15 min for all samples. The 15 min pretreatment reduced hard seed from 53 to 2% and increased germination from 36% to 85% on average for all samples. This same pretreatment also reached germination to 64% and 79% after 2 and 4 days incubation respectively, compared to 3% and 10% for untreated seeds. The seed imbibition percentage at 24 hour correlated significantly (at 1% level) with the final germination percentage at 14 days for each species.

## Introduction

Seed dormancy is of agricultural and ecological importance. It can lengthen the longevity of seeds and has a large impact on the survival and emergence of plants in the wild *(Ellis et al.,* 1985; Argel and Paton, 1999). However, it also affects the uniformity of germination of crops during field establishment, and poses a major challenge in seed testing and genebank operations as dormant seed will not germinate even under optimum conditions. Seeds of many wild species of legumes are most likely to have hard seed, and pretreatments for dormancy breaking are required before testing and sowing (ISTA, 2003). Much research has been done on breaking dormancy of hard seeds of temperate legumes (Bradbeer, 1988), but work on tropical and subtropical forage species has been limited (Argel and Paton, 1999).

Pretreatment with concentrated sulfuric acid is one of the mostly widely applied methods to remove hardseededness (Argel and Paton, 1999). The method is also effective in controlling seed borne fungi at the same time as breaking hard seed dormancy (Nan *et al.,* 1998). The pretreatment causes degradation of the seed coat thus allowing greater permeability to water (Nagaveni and Srimathi, 1980). The times used for pretreatment

with sulfuric acid vary from a few seconds to several hours, and in the majority of cases pretreatment periods are between 1 and 20 min (Ellis *et al., 1985).* Concentrated sulfuric acid scarification for breaking seed dormancy for some forage legumes are also recommended by the International Seed Testing Association (ISTA) rules, and include *Desmodium intortum, D. uncinatum, Macroptilium atropurpreum, M. lathyroides, Macrotyloma axillare, Pueraria phaseoloides* and *Stylosanthes guianensis* (ISTA, 2004).

The genus *Vigna* contains a number of important tropical and subtropical agricultural species (Vanderborght, 1989; Lawn and Watkinson, 2002). A high content of hard seeds with variation between lines and accessions has been reported in many species (Pentney *et al.,* 1984; Tomer and Kumari Promila, 1991; Wang *et al.,* in preparation). The suitability of techniques that have been widely and successfully employed for breaking dormancy of hard seeds of other legumes were studied for different accessions of 5 wild *Vigna* species, which are represented by several accession in the forage germplasm collection maintained in the ILRI genebank but whose seed dormancy characteristics were unknown. The species included *V. membranacea A.* Rich., *V. oblongifolia A.* Rich., V. *racemosa* (G. Don) Hutch. and Dalziel, *V. schimperi* Baker and V. *vexillata A.* Rich. The effects of hot water and mechanical scarification on dormancy breaking of seeds of these species have been reported separately *(Wang et al.,* in preparation). This paper addresses only the effect of different periods of pretreatment with concentrated sulfuric acid in order to break dormancy due to hard seededness in these species.

## Materials and methods

### *Seed Material*

Seed samples of 18 lots of 14 accessions of *Vigna* species were, selected for the study based on year of harvest (table 1). The seeds had previously been harvested from regeneration plots at Debre Zeit or Soddo; research sites located at mid altitude in the Ethiopian Rift Valley in areas where the germplasm was adapted. After harvest, seeds were dried to a moisture content of 5% and stored in sealed aluminum foil bags at *8°C* in the genebank for periods varying from 3 to 8 years,

### *Scarification pretreatment*

Two hundred seeds from each seed lot were immersed in 25 ml concentrated sulfuric acid for 3, 6, 9 12 or 15 min, after which seeds were rinsed thoroughly with tap water six times, and then dried on top of filter paper for 24 hours on the laboratory bench. Non scarified seeds were used as the control.

### *Germination test and data recording*

After each pretreatment, four replicates of 50 seed from each sample were plated on top of 2 layers of filter paper in 90mm petri dishes, which were randomly arranged and incubated at 25°C in an incubator without light for a period of 14 days. The following parameters were recorded:

*Imbibition rate:* Number of seed imbibed was counted after 24 hours of incubation and recorded as percentage of seed imbibed.

*Germination rate, final germination percentage, percentages of hard seed and dead seed:* The germinating seeds were checked daily (± 2 hour) and any germinated seedlings were recorded and removed. A seed was considered to have germinated when the radical extension was at least 0.5 cm and the seedling was normal (ISTA, 2004). At the end of the germination test, normal seedlings, hard seed, abnormal seedlings, fresh ungerminated seed and dead seed were evaluated according to the ISTA Rules (ISTA, 2004) and the percentage of seeds in each category were calculated. Daily accumulated germination percentages were also calculated to assess germination rate.

*Data analysis*

Data were analyzed with SAS version 9.1 (SAS Institute, 2003). All percentage data were Arcsine transformed before being subjected to LSD test for comparison among the pretreatments at P = 0.05 level. Correlation and regression analyses were applied to assess the relationship between the imbibition rate and the final germination percentage for each species.

**Table 1. Background information of species, accessions and seed lots tested.**

| Species | Accession / lot | Country of collection | Seed production site in Ethiopia | Year harvested | Plant type |
|---|---|---|---|---|---|
| *V. membranacea* | 7534 / 5 | Ethiopia | Debre Zeit | 1999 | Annual |
| | 7899 / 3 | Ethiopia | Debre Zeit | 1999 | Annual |
| | 10525 / 6 | Ethiopia | Debre Zeit | 1999 | Annual |
| *V. oblongifolia* | 30 / 4 | Nigeria | Debre Zeit | 1999 | Annual |
| | 38 / 2 | Nigeria | Soddo | 1998 | Annual |
| | 39 / 3 | Nigeria | Debre Zeit | 1999 | Annual |
| | 7708 / 5 | Ethiopia | Debre Zeit | 1999 | Annual |
| *V. racemosa* | 45 / 2 | Nigeria | Soddo | 1998 | Perennial |
| | 45 / 4 | Nigeria | Soddo | 1999 | Perennial |
| | 47 / 4 | Nigeria | Soddo | 1999 | Perennial |
| | 47 / 5 | Nigeria | Soddo | 2000 | Perennial |
| *V. schimperi* | 10530 / 11 | Ethiopia | Debre Zeit | 2001 | Perennial |
| | 10530 / 12 | Ethiopia | Debre Zeit | 2002 | Perennial |
| | 10530 / 13 | Ethiopia | Debre Zeit | 2002 | Perennial |
| *V. vexillata* | 61 / 2 | Nigeria | Soddo | 1998 | Perennial |
| | 67 / 2 | Nigeria | Debre Zeit | 1999 | Perennial |
| | 7901 / 4 | Ethiopia | Debre Zeit | 1999 | Perennial |
| | 13264 / 5 | Ethiopia | Debre Zeit | 1999 | Perennial |

## Results

*Hard seed*

All pretreatments significantly ($P < 0.05$) reduced the percentage of hard seeds as compared to non treated control seeds except for the 3 min pretreatment for accession 7708/5 (table 2). The percentage of hard seed decreased gradually as the pretreatment duration increased for all samples. The longest duration pretreatment of 15 min reduced the hard seed percentage to an average of 2% compared to an initial average of 53%.

*Germination percentage*

The final germination percentage of all accessions, with a few exceptions, was significantly increased ($P < 0.05$) by the pretreatments (figure 1). The total increase on average was 42 percent point for all samples (from 36% of control to 78% of pretreatments). The exceptions were accessions 7708/5, 45/2, 45/4 and 7901/4 in which germination was not

Table 2. Effect of sulfuric acid scarification pretreatments of varying lengths of time, on breaking hard seed dormancy in 18 accessions and seed lots of five *Vigna* species.

| Species | Accession / lot | Percentage of hard seed | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | 3 min | 6 min | 9 min | 12 min | 15 min |
| *V. membranacea* | 7534 / 5 | 75$^{a}$ | 12$^{b}$ | 3$^{c}$ | 1$^{cd}$ | 1$^{cd}$ | 0$^{d}$ |
| | 7899 / 3 | 91$^{a}$ | 29$^{b}$ | 17$^{c}$ | 16$^{c}$ | 4$^{d}$ | 5$^{d}$ |
| | 10525 / 6 | 58$^{a}$ | 22$^{b}$ | 8$^{c}$ | 3$^{d}$ | 2$^{d}$ | 1$^{d}$ |
| *V. oblongifolia* | 30 / 4 | 41$^{a}$ | 20$^{b}$ | 12$^{c}$ | 3$^{d}$ | 0$^{e}$ | 0$^{e}$ |
| | 38 / 2 | 82$^{a}$ | 74$^{b}$ | 70$^{bc}$ | 63$^{c}$ | 43$^{d}$ | 28$^{e}$ |
| | 39 / 3 | 69$^{a}$ | 35$^{b}$ | 11$^{c}$ | 3$^{d}$ | 1$^{de}$ | 0$^{e}$ |
| | 7708 / 5 | 18$^{a}$ | 19$^{a}$ | 6$^{b}$ | 1$^{c}$ | 0$^{c}$ | 0$^{c}$ |
| *V. racemosa* | 45 / 2 | 22$^{a}$ | 11$^{b}$ | 8$^{b}$ | 0$^{c}$ | 0$^{c}$ | 0$^{c}$ |
| | 45 / 4 | 51$^{a}$ | 38$^{b}$ | 8$^{c}$ | 1$^{d}$ | 0$^{d}$ | 0$^{d}$ |
| | 47 / 4 | 99$^{a}$ | 75$^{b}$ | 21$^{c}$ | 2$^{d}$ | 1$^{cd}$ | 0$^{d}$ |
| | 47 / 5 | 72$^{a}$ | 41$^{b}$ | 22$^{c}$ | 2$^{d}$ | 3$^{d}$ | 0$^{e}$ |
| *V. schimperi* | 10530 / 11 | 39$^{a}$ | 17$^{b}$ | 15$^{b}$ | 6$^{c}$ | 2$^{d}$ | 2$^{d}$ |
| | 10530 / 12 | 35$^{a}$ | 20$^{b}$ | 9$^{c}$ | 6$^{cd}$ | 4$^{cd}$ | 2$^{d}$ |
| | 10530 / 13 | 65$^{a}$ | 25$^{b}$ | 17$^{b}$ | 6$^{c}$ | 2$^{d}$ | 1$^{d}$ |
| *V. vexillata* | 61 / 2 | 77$^{a}$ | 52$^{b}$ | 43$^{b}$ | 24$^{c}$ | 9$^{d}$ | 4$^{e}$ |
| | 67 / 2 | 18$^{a}$ | 9$^{b}$ | 6$^{b}$ | 4$^{bc}$ | 2$^{dc}$ | 0$^{d}$ |
| | 7901 / 4 | 14$^{a}$ | 6$^{b}$ | 2$^{c}$ | 0$^{d}$ | 0$^{d}$ | 1$^{cd}$ |
| | 13264 / 5 | 29$^{a}$ | 6$^{b}$ | 2$^{c}$ | 0$^{d}$ | 0$^{d}$ | 1$^{d}$ |
| | Average | 53 | 28 | 16 | 8 | 4 | 2 |

Means within a row followed by the different letter are significantly different at 5% level.

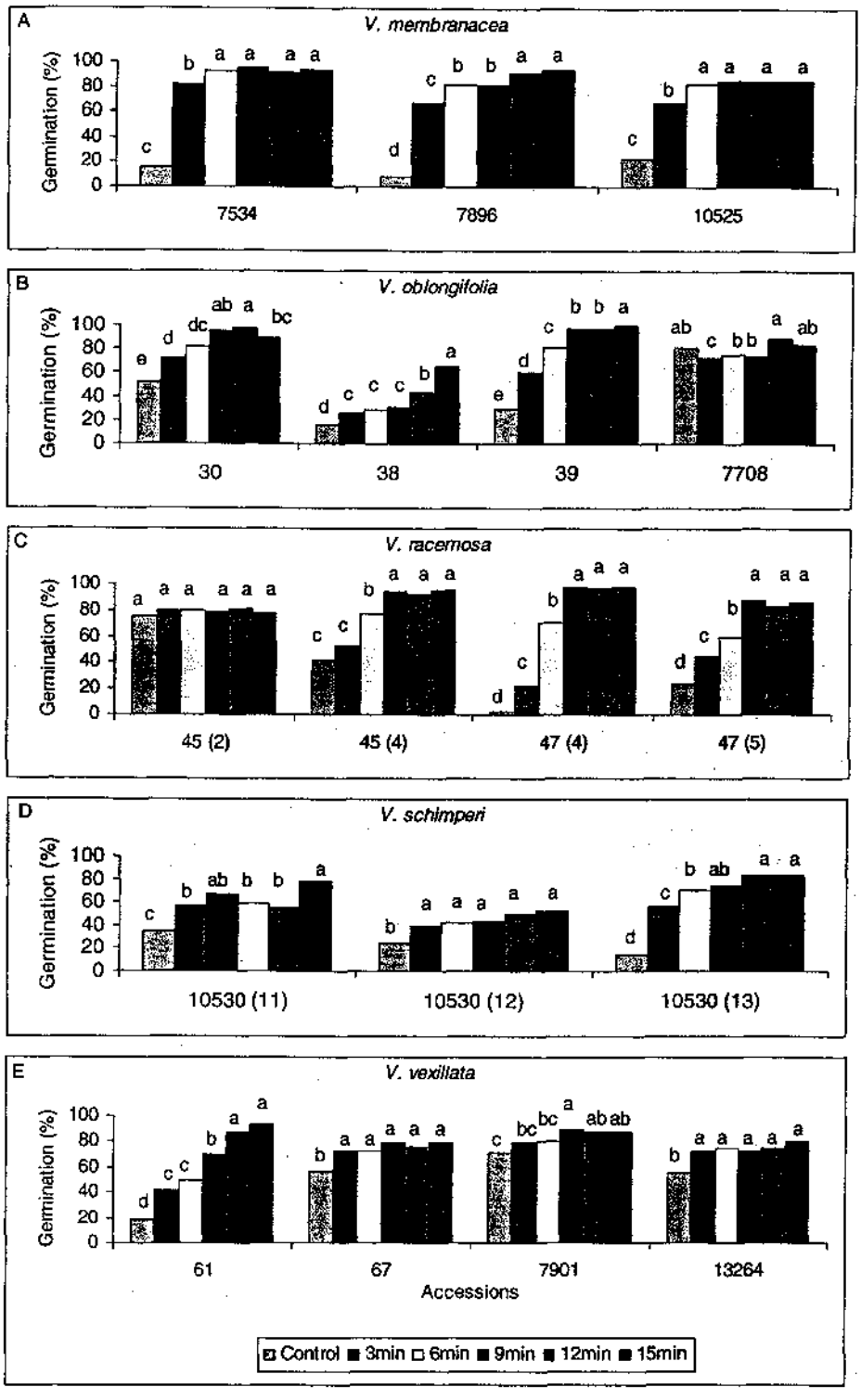


Figure 1. Effect of sulfuric acid pretreatments on final germination percentage of different accessions of five *Vigna* species. Means within a seed sample marked by different letters are significantly different at the 5% level.

promoted by all pretreatment periods. Most of these accessions though, began with higher initial germination percentages, and dormancy was therefore not nearly as pronounced as with other seed lots.

*Abnormal seedlings and dead seeds*

For the majority of the seed lots, when compared to the control, the pretreatments did not increase the percentage of abnormal seedlings / dead seeds ($P > 0.05$). However, exceptions were samples 7708/5, 45/2 and 47/5, where the number of abnormal seedlings / dead seeds was significantly increased ($P < 0.05$) by all pretreatments, and the extent of the increase was not significantly ($P < 0.05$) related to the duration of the pretreatment. The average increase across the pretreatments was from 1% (control) to 18%, from 2% to 17% and from 3% to 14% for the samples 7708/5, 45/2 and 47/5, respectively (table 3).

Table 3. Effect of sulfuric acid scarification pretreatments of varying time periods on percentage of total abnormal seedling / dead seed in 18 accessions and seed lots of five *Vigna* species.

| Species | Accession / lot | Abnormal seedling / dead seed after pretreatments | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | 3 min | 6 min | 9 min | 12 min | 15 min |
| *V. membranacea* | 7534 / 5 | 9 | ns | ns | ns | ns | ns |
| | 7899 / 3 | 1 | ns | ns | ns | ns | ns |
| | 10525 / 6 | 20 | ns | ns | ns | ns | ns |
| *V. oblongifolia* | 30 / 4 | 8 | ns | ns | ns | ns | ns |
| | 38 / 2 | 1 | ns | ns | 6 | 11 | 6 |
| | 39 / 3 | 2 | ns | ns | ns | ns | ns |
| | 7708 / 5 | 1 | 18 | 18 | 25 | 12 | 17 |
| *V. racemosa* | 45 / 2 | 2 | 9 | 13 | 21 | 20 | 24 |
| | 45 / 4 | 2 | ns | 10 | ns | ns | ns |
| | 47 / 4 | 0 | ns | ns | ns | ns | ns |
| | 47 / 5 | 3 | 15 | 14 | 11 | 15 | 14 |
| *V. schimperi* | 10530 / 11 | 26 | ns | ns | 35 | 42 | ns |
| | 10530 / 12 | 37 | ns | ns | ns | ns | ns |
| | 10530 / 13 | 18 | ns | ns | ns | ns | ns |
| *V. vexillata* | 61 / 2 | 4 | ns | ns | ns | ns | ns |
| | 67 / 2 | 25 | ns | ns | ns | ns | ns |
| | 7901 / 4 | 14 | ns | ns | ns | ns | ns |
| | 13264 / 5 | 17 | ns | ns | 27 | ns | ns |

Percentage data is only showed for those pretreatments that were significantly different from the control ($P < 0.05$). The remaining data were not significant at $P > 0.05$ (ns)

*Germination rate*

All pretreatments increased the germination rate markedly. The cummulative germination of treated seeds increased rapidly and reached maximum levels well before the control (figure 2). The germination rate improved gradually as the pretreatment time period increased from 3 to 15 min, with the best results obtained from the 15min pretreatment. The time to 50% germination ($T_{50}$) was 2 days for seeds treated with sulfuric acid for 15min in contrast to the control seeds, which had not yet reached $T_{50}$ at the end of the test, which was after 14 days.

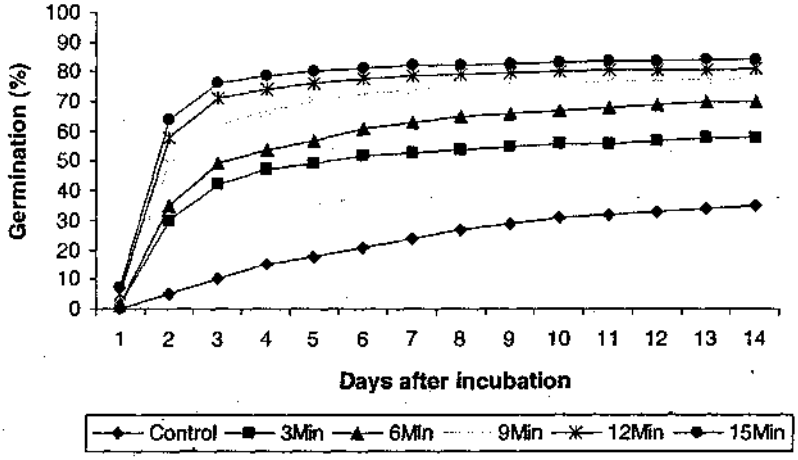


**Figure 2. Effects of a number of sulfuric acid pretreatments on mean germination rate (cummulative germination) across all five *Vigna* species**

*Relationship between imbibition rate and final germination*

The logarithmic transformed regression model for imbibition rate was fitted to the data. The imbibition rate correlated positively to the final germination percentage over the accessions or samples tested for each species (figure 3). All correlations were significant at 1% level with R ranging from 0.6885 to 0.9080.

## Discussion

This study demonstrated that a pretreatment with concentrated sulfuric acid for15 min was highly effective in breaking dormancy in the majority of *Vigna* species, accessions and seed lots tested. The effectivity of the pretreatment declined as the pretreatment period decreased from 15 to 3 min (table 2, figure 1 and figure 2). This supports the previous findings that the pretreatment length when using sulfuric acid is critical (Ellis *et al.,* 1985; Teketay 1996; Rincon-Rosales *et al.,* 2003; Emongor *et al.,* 2004). Pretreatment periods should ideally be between one and twenty minus (Ellis *et al.,* 1985) in order to be effective. A 15min pretreatment reduced hard seed from 53% to 2% and increased final germination from 36% to 85%, (table 2, figure 1). The 15min pretreatment did not substantially increase the number of dead or abnormal seeds, and so cannot be considered

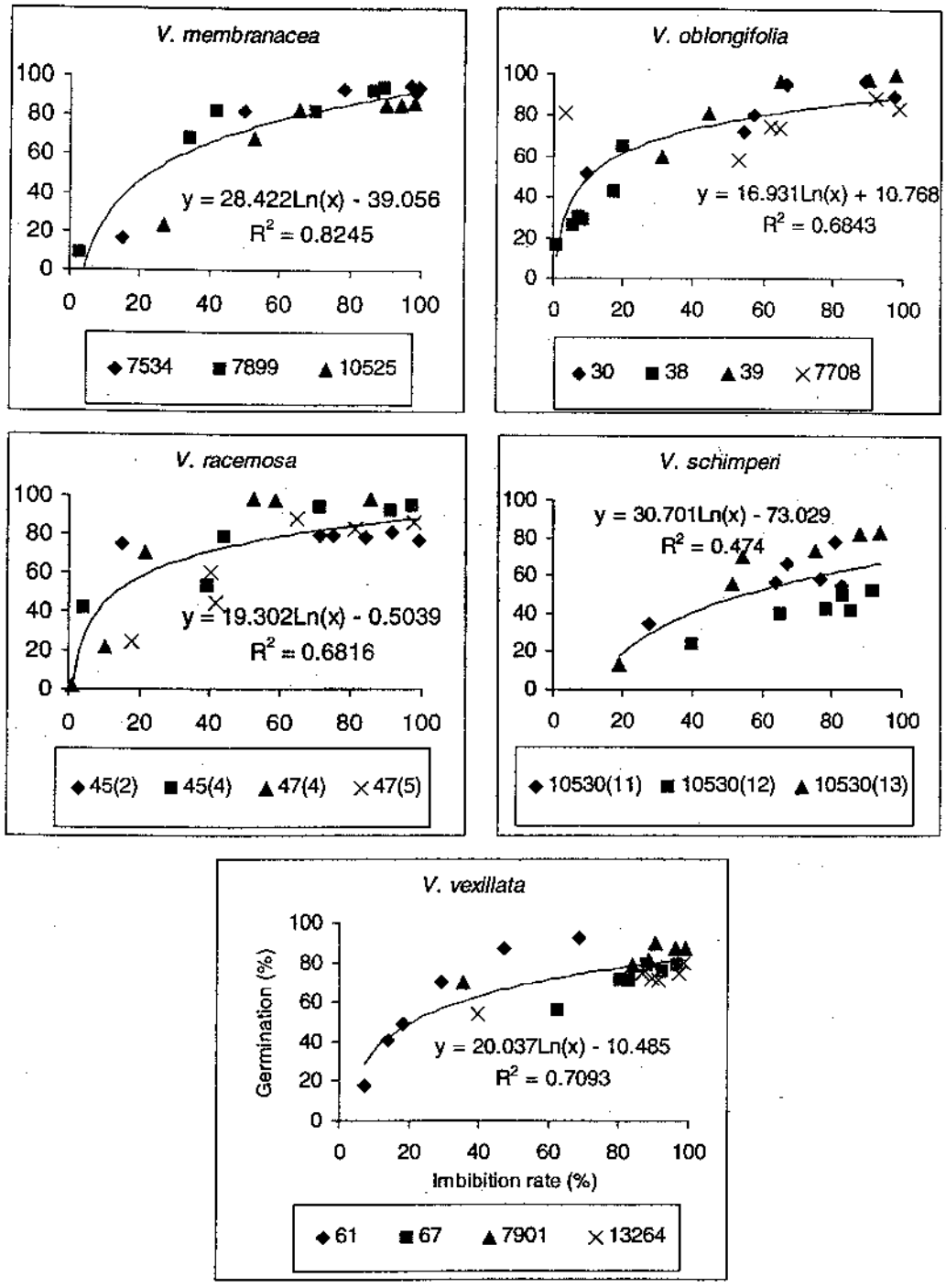


Figure 3. Relationship between imbibition rates at 24 hours and final germination after 14 days of incubation of five *Vigna* species.

harmful. The pretreatment also dramatically increased the germination rate, with maximum germination reached after 4 days in the majority of seed samples tested when compared to *10%* of the control (figure 2). This was a similar rate to that obtained for the same seed samples by mechanically scarifying the seed coat (Wang *et al.,* in preparation). This indicates that extending the treatment period beyond 15min is unlikely to be helpful in improving the effectivity of the test. The 15 min sulfuric acid pretreatment was superior to a hot water pretreatment (80°C for 6 min) and sand paper scarification for the same seed samples tested *(Wang et al.,* in preparation). Similar results on the suitability of sulfuric acid pretreatments have also been reported for seeds of other *Vigna* species like *V. radiata* (Verma and Singh, 1989), V. *mungo* (Tomer and Kumari Promila, 1991) and *V. umbellata* (Tomer and Singh, 1993) and some other legumes (Konda, 1993; Pandita- *et al.,* 1999; Das and Saha, 1999; Rincon-Rosales *et al.,* 2003).

It has been reported that sulfuric acid pretreatment have the potential to kill seeds in accessions or populations (Ellis *et al.,* 1985) and similar damage was found for three out of eighteen accessions/lots tested in this study (table 3). The damage was not however, related to the period of the pretreatment, which indicates that some seed lots are perhaps more sensitive to the concentrated acid pretreatment and alternative methods need be considered for these samples.

The imbibition rate was significantly ($P = 0.01$) positively correlated to the final germination percentage for each species (figure 3) and, a similar phenomenum was observed for the same seed samples when other scarification techniques were applied *(Wang et al.* in preparation). This raises the possibility of using the imbibition as a rapid test for screening seed dormancy breaking techniques in the laboratory.

In conclusion, a 15min pretreatment of concentrated sulfuric acid is recommended for breaking hard seed dormancy prior to germination testing of *Vigna membranacea, V. oblongifolia, V. racemosa, V. schimperi* and V. *vexillata.* The method can be applied during laboratory testing or prior to planting for regeneration from genebanks since many seeds can be treated at once. However, given the variation in response to the pretreatment by the diverse accessions maintained in a genebank, particular care should be taken to dedtermine whether any of the treated seed lots have lower levels of hard seed dormancy or are sensitive to the pretreatment. In these cases it would be preferable to use mechanical scarification to avoid damage to the seeds and false results in germination tests.

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