Quantification of Total and Soluble Inorganic Phosphate

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[Abstract] A simple, rapid, and sensitive colorimetric microassay for inorganic phosphate (Pi) relies upon the absorption at 660 nm of a molybdenum blue complex that forms upon reduction of an ammonium molybdate-Pi complex in acid. The method for determination of total Pi uses plant tissues that have been ashed at 500 °C, whereas quantification of soluble Pi is performed with tissues extracted under mild acid conditions (which preserves acid-labile phosphate ester bonds).

Materials and Reagents

1. Plant tissues
2. 17.5 M Glacial acetic acid
3. 12 M Concentrated HCl
4. 16 M Concentrated HNO_3
5. Sodium phosphate, monobasic (NaH_2PO_4) (Bioshop, catalog number: SPM400)
6. Ascorbic acid (Bioshop, catalog number: AS0704)
7. Ammonium molybdate (Bioshop, catalog number: AMN333)
8. Zinc acetate (Sigma-Aldrich, catalog number: Z0625)
9. Quartz crucibles (Thermo Fisher Scientific, catalog number: 08-072 series)
10. Acid extraction solution (see Recipes)
11. Pi stock (for standard curve) (see Recipes)
12. Pi assay reagent (see Recipes)

Equipment

1. Crucible
2. Drying oven
3. Repeat pipetor
4. Isotemp Muffle Furnace (Thermo Fisher Scientific, model: 10-650-14)
5. Microcentrifuge
6. A computer supported microplate spectrophotometer (*e.g.*, Spectromax Plus, Molecular Devices, Sunnyvale, CA, U.S.A.)

Procedure

A. Total Pi (Hurley *et al*., 2010)

1. Acid wash crucibles by incubating for at least 1 h in 0.1 N HCl at room temperature, then rinse with dH_2O and dry.
2. Pre-weigh crucibles and place at least 60 mg (fresh weight) of tissue in each.
3. Dry in oven at 50-80 °C for at least 16 h (*e.g.,* overnight), and then record tissue's dry weight (mg) in each crucible.
4. Ash the tissue in the furnace using a temperature ramp program (20 min at 150 °C, 1 h at 250 °C, and 3 h at 500 °C).
5. Weigh crucible and ash. Add 25 µl of acid extraction solution per mg of ash, mix well, and centrifuge at 11,000 *x g* for 10 min.
6. Dilute the supernatant 50-fold in dH_2O.
7. Assay Pi using the Drueckes *et al*. (1995) protocol as modified for plant tissues (Bozzo *et al*., 2006) by preparing a standard curve over the range 1-133 nmol of Pi using the following template.

Well #	Vol of Pi stock (3.3 mM)	Volume of dH_2O	Amount of Pi added
	(µl)	(µl)	(nmol)
1A	0	40	0
1B	2	38	6.6
1C	4	36	13.2
1D	8	32	26.4
1E	12	28	39.6
1F	16	24	52.8
1G	20	20	66.0
1H	24	16	79.2
2A	30	10	99.9
2B	35	5	116.6
2C	40	0	133.2

   a. Pipette 1-40 µl of unknown(s) into adjacent wells(s). Add dH_2O to bring each well to 40 µl final volume.
   b. Add 200 µl of Pi assay reagent to each well using a repeat pipetor.
   c. Incubate at 37 °C for 30 min.
   d. Measure A_{660} values and use the Pi calibration (standard) curve to determine Pi content of unknowns.
   e. Express the data as: nmol Pi mg^{-1} dry weight.

B. Soluble Pi (Bozzo *et al*., 2006)
   1. Extract snap-frozen tissues (1:5, w/v) with 1% (v/v) glacial acetic acid.
   2. Centrifuge samples at 11,000 *x g* for 10 min.
   3. Assay the supernatant for Pi as described above.
   4. Esterified-Pi is calculated from the difference between total and free Pi concentrations.

Recipes

1. Acid extraction solution
   30 ml 12 M concentrated HCl
   10 ml 16 M concentrated HNO_3
   60 ml dH_2O
2. Pi stock (for standard curve)
   3.3 mM NaH_2PO_4
3. Pi assay reagent
   a. Ammonium molybdate reagent
   b. Ammonium molybdate is added to an aqueous solution of 15 mM zinc acetate to give a 10 mM solution of molybdate. The solution is then adjusted to pH 5.0 with HCl. This solution is stored at 4 °C in the dark and is stable for several months.
   c. Reducing reagent
      A 10% (w/v) solution of ascorbic acid is adjusted to pH 5.0 with NaOH.
      Note: This solution must be prepared fresh daily.
   d. The Pi assay reagent is prepared by mixing one part of the ammonium molybdate reagent with four parts of the reducing reagent (prepare fresh daily).

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References

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